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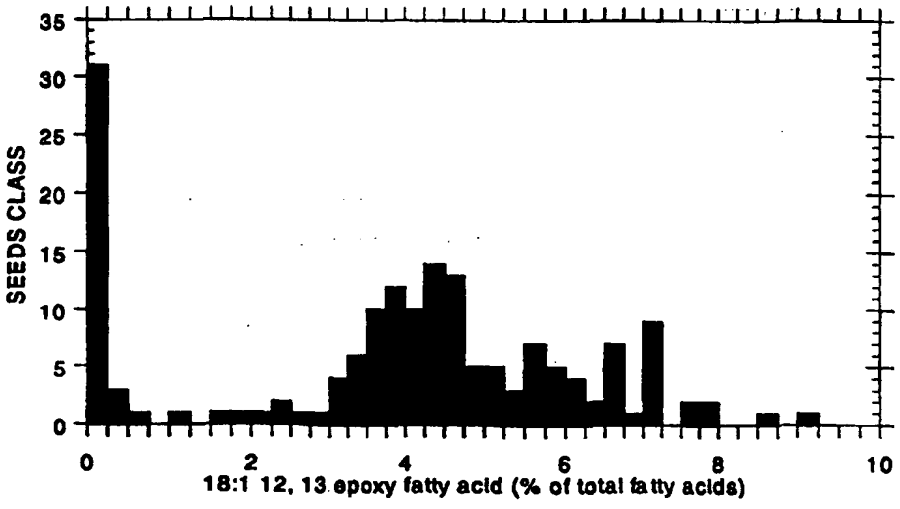
(57) Abstract

This invention relates to isolated nucleic acid fragments encoding all or a substantial portion of *Vernonia galamenensis* fatty acid modifying enzymes. The invention also relates to the construction of chimeric genes encoding all or a portion of *Vernonia galamenensis* fatty acid modifying enzymes, in sense or antisense orientation, wherein expression of the chimeric gene results in production of altered levels of a *Vernonia galamenensis* fatty acid modifying enzymes in transformed host cells.



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TITLE

FATTY ACID MODIFYING ENZYMES FROM DEVELOPING SEEDS OF *VERNONIA GALAMENENSIS*

FIELD OF THE INVENTION

This invention is in the field of plant molecular biology. More specifically, this invention pertains to nucleic acid fragments encoding enzymes involved in fatty acid biosynthesis and modification in plants and seeds.

BACKGROUND OF THE INVENTION

Fatty acids bearing chemical modifications in addition to the common double bonds are found in the storage lipids of many oilseeds (Harwood, J. L. (1980) *In* The Biochemistry of Plants, T.S. Moore Jr., *ed.* CRC Press, New York, pp 91-116). Some of these modifications functionalize the fatty acid to produce products that are useful in industrial applications; this is opposed to the more common usage of plant-derived lipids as foods. Examples are the use of the hydroxylated fatty acid ricinoleic acid in lubricants, and the short- or medium-carbon chain length fatty acids from palm oil in detergents. In some cases, fatty acid composition of the storage lipids of oilseeds produced in temperate climates can be modified by the addition of genes from exotic sources so that large amounts of unique fatty acids are produced (Ohlrogge, J. B. (1994) *Plant Physiol. 104*, 821-826).

Epoxidation is among the known modifications to storage lipid fatty acids. The 18-carbon fatty acid 9,10-ene-12,13-epoxide comprises as much as 60% of the total seed fatty acid in species such as *Vernonia galamenensis* and *Euphorbia lagascea* (Bafor, M. et al. (1993) *Arch. Biochem Biophys 303*:145-151). Fatty acids carrying the epoxide modification may find use as plasticizers, in crosslinking coatings applications, and in setting printing inks.

Attempts to delineate the biosynthetic pathway of cis-12-epoxyoctadeca-cis -9-enoate indicate that the catalytic activity responsible for the introduction of the epoxide moiety is in the microsomal membrane fraction, most likely the endoplasmic reticulum (Bafor et al. *supra*). While the above study also suggests that the catalytic activity responsible is an enzyme in the cytochrome P450 mono-oxygenase class, enzymes with amino acid sequences related to the endoplasmic reticulum-localized fatty acid desaturases have been isolated from tissues that produce hydroxylated fatty acids (World Patent Publication No. WO94/11516). These sequences have been shown to be active in adding the hydroxyl group to esterified fatty acids (Broun, P. and Somerville, C. (1997) *Plant Physiol 113*: 933-942). It is therefore possible that fatty acid epoxidizing enzymes may be related in sequence to the class of membrane bound enzymes responsible for fatty acid desaturation and fatty acid hydroxylation.

Thus while candidate enzyme classes have been suggested, no gene sequences from those candidate classes and from tissues that are known to produce epoxidized fatty acids have been isolated.

SUMMARY OF THE INVENTION

The instant invention relates to isolated nucleic acid fragments encoding plant enzymes involved in fatty acid biosynthesis and modification, the enzymes having sequence homology to membrane-bound fatty acid desaturases. Specifically, this invention concerns isolated nucleic acid fragments encoding a fatty acid epoxidizing enzyme and a fatty acid desaturase enzyme, each of which are normally expressed in developing seeds of *Vernonia galamenensis*. In addition, this invention relates to nucleic acid fragments that are complementary to nucleic acid fragments encoding the *Vernonia galamenensis* fatty acid epoxidizing and desaturase enzymes.

In another embodiment, the instant invention relates chimeric genes that comprise nucleic acid fragments encoding *Vernonia galamenensis* fatty acid epoxidizing or desaturase enzymes operably linked to suitable regulatory sequences, wherein expression of the chimeric genes results in production of levels of the encoded protein in transformed host cells. For example, disclosed herein is a chimeric gene wherein a nucleic acid fragment encoding a *Vernonia galamenensis* fatty acid epoxidizing enzyme or a *Vernonia galamenensis* fatty acid desaturase enzyme is operably linked to one or more regulatory sequences suitable for directing expression of the *Vernonia galamenensis* fatty acid epoxidizing or desaturase enzymes in microbial cells.

In a further embodiment, the instant invention concerns a transformed host cell comprising in its genome a chimeric gene comprising a nucleic acid fragment encoding a *Vernonia galamenensis* fatty acid epoxidizing or desaturase enzyme operably linked to suitable regulatory sequences. Expression of the chimeric gene results in production of altered levels of protein encoded by the operably linked nucleic acid fragment in the transformed host cell. The transformed host cell can be of eukaryotic or prokaryotic origin, and include cells derived from higher plants and microorganisms. The invention also includes transformed plants that arise from transformed host cells of higher plants, and from seeds derived from such transformed plants.

A further embodiment of the instant invention is a soybean seed that accumulates vernolic acid.

An additional embodiment of the instant invention concerns a method of altering the level of expression of a *Vernonia galamenensis* fatty acid epoxidizing enzyme or a *Vernonia galamenensis* fatty acid desaturase enzyme in a transformed host cell comprising:
a) transforming a host cell with a chimeric gene comprising a nucleic acid fragment encoding a *Vernonia galamenensis* fatty acid epoxidizing enzyme or a *Vernonia galamenensis* fatty acid desaturase enzyme; and b) growing the transformed host cell under conditions that are

suitable for expression of the chimeric gene wherein expression of the chimeric gene results in production of altered levels of protein encoded by the operably linked nucleic acid fragment in the transformed host cell.

Yet another embodiment of the instant invention concerns a method for producing epoxidized fatty acids in the seeds of plants comprising the following steps: (a) transforming a plant cell with a chimeric gene encoding all or a portion of the *Vernonia galamenensis* fatty acid epoxidizing enzyme operably linked in sense orientation to suitable regulatory sequences; (b) growing a fertile mature plant from the transformed plant cell of step (a) under conditions suitable to obtain seeds; and (c) selecting from the progeny seed of step (b) those seeds containing epoxidized fatty acids.

Another embodiment of the instant invention is a method for producing *Vernonia galamenensis* fatty acid epoxidizing enzyme or *Vernonia galamenensis* fatty acid desaturase enzyme comprising the following steps: (a) transforming a microbial host cell with a chimeric gene wherein a nucleic acid fragment encoding a *Vernonia galamenensis* fatty acid epoxidizing enzyme or a *Vernonia galamenensis* fatty acid desaturase enzyme is operably linked to regulatory sequences suitable for directing expression in microbial cells; and (b) growing the transformed microbial cells obtained from step (a) under conditions that result in expression of the *Vernonia galamenensis* fatty acid epoxidizing or desaturase enzyme proteins.

An additional embodiment of the instant invention concerns a method for obtaining a nucleic acid fragment encoding all or substantially all of an amino acid sequence encoding *Vernonia galamenensis* fatty acid epoxidizing or desaturase enzymes.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following detailed description and the accompanying drawing and sequence descriptions which form a part of this application.

Figure 1 shows a comparison of the amino acid sequences of the instant *Vernonia galamenensis* fatty acid desaturase (vs1.05h08), the instant *Vernonia galamenensis* fatty acid epoxidase (vs1.02c07), a soybean fatty acid desaturase (soy) and a castor bean fatty acid hydroxylase (castor). The eight conserved histidine residues found in all membrane bound fatty acid modifying enzymes of this class are boxed and stippled. An arginine residue found in the fatty acid hydroxylase from castor bean and in the instant *Vernonia galamenensis* fatty acid epoxidase but not in the fatty acid desaturating enzymes of this class is also boxed and stippled. Other residues in the *Vernonia* fatty acid epoxidase sequence that are unique to that sequence but conserved in most or all of the other sequences are boxed. Not all amino acid changes are marked: only those in which the change in the epoxidase sequence occurs in a highly conserved region and in which the amino acid present in the

epoxidase is different in physical character from the conserved residues in the other sequences are marked.

Figure 2 is a restriction map of plasmid pRVF7 used for soybean transformations.

Figure 3 is a histogram of the results of single seed analyses of the accumulation of vernolic acid in seeds from plants transformed with pRVF7.

The following sequence descriptions and sequence listings attached hereto comply with the rules governing nucleotide and/or amino acid sequence disclosures in patent applications as set forth in 37 C.F.R. §1.821-1.825.

SEQ ID NO:1 is the nucleotide sequence comprising the cDNA insert in clone vs1.05h08 encoding a *Vernonia galamenensis* fatty acid desaturase enzyme.

SEQ ID NO:2 is the deduced amino acid sequence of a *Vernonia galamenensis* fatty acid desaturase enzyme derived from the nucleotide sequence of SEQ ID NO:1.

SEQ ID NO:3 is the nucleotide sequence comprising the cDNA insert in clone vs1.02c07 encoding a *Vernonia galamenensis* fatty acid epoxidizing enzyme.

SEQ ID NO:4 is the deduced amino acid sequence of a *Vernonia galamenensis* fatty acid epoxidizing enzyme derived from the nucleotide sequence of SEQ ID NO:3.

SEQ ID NO:5 is the amino acid sequence encoding the soybean (*Glycine max*) fatty acid desaturase enzyme depicted in Figure 1 and having GenBank Accession No. L43920.

SEQ ID NO:6 is the amino acid sequence encoding the castor bean (*Ricinus communis*) fatty acid hydroxylase enzyme depicted in Figure 1 and having GenBank Accession No. U22378.

SEQ ID NO:7 shows the nucleotide sequence of the PCR primer used as the 5' end primer in PCR reactions for amplification of the coding region of vs1.02c07.

SEQ ID NO:8 shows the nucleotide sequence of the PCR primer used as the 3' end primer in PCR reactions for amplification of the coding region of vs1.02c07.

SEQ ID NO:9 shows the nucleotide sequence of the PCR primer used as the 5' end primer in PCR reactions for amplification of the coding region of vs1.05h08.

SEQ ID NO:10 shows the nucleotide sequence of the PCR primer used as the 3' end primer in PCR reactions for amplification of the coding region of vs1.05h08.

The Sequence Descriptions contain the one letter code for nucleotide sequence characters and the three letter codes for amino acids as defined in conformity with the IUPAC-IYUB standards described in *Nucleic Acids Research 13*:3021-3030 (1985) and in the *Biochemical Journal 219 (No. 2)*:345-373 (1984) which are herein incorporated by reference. The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

BIOLOGICAL DEPOSIT

The following soybean seed has been deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, and bears the following designation, accession number and date of deposit.

Designation	Accession Number	Date of Deposit
798	ATCC XXXXX	June __, 1998

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to isolated nucleic acid fragments encoding all or a substantial portion of fatty acid epoxidizing and desaturase enzymes normally expressed in developing seeds of *Vernonia galamenensis* that are similar in sequence to other plant, membrane-bound fatty acid desaturases. The invention also relates to the construction of a chimeric gene comprising an nucleic acid fragment encoding all or a portion of the *Vernonia galamenensis* fatty acid epoxidizing or desaturase enzyme, operably linked in sense or antisense orientation to suitable regulatory sequences, wherein expression of the chimeric gene results in production of altered levels of the desired enzyme in a transformed host cell. The invention also relates to methods of using isolated nucleic acid fragments encoding all or a substantial portion of the fatty acid epoxidizing and desaturase enzymes normally expressed in developing seeds of *Vernonia galamenensis*.

In the context of this disclosure, a number of terms shall be utilized. As used herein, an "isolated nucleic acid fragment" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

The term "*Vernonia galamenensis* fatty acid modifying enzyme" refers collectively to the *Vernonia galamenensis* fatty acid epoxidizing enzyme and the *Vernonia galamenensis* fatty acid desaturase enzyme disclosed in the instant specification.

As used herein, "substantially similar" refers to nucleic acid fragments wherein changes in one or more nucleotides results in substitution of one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. "Substantially similar" also refers to nucleic acid fragments wherein changes in one or more nucleotides does not affect the ability of the nucleic acid fragment to mediate alteration of gene expression by antisense or co-suppression technology. "Substantially similar" also refers to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially affect the functional properties of the resulting transcript vis-à-vis the ability to mediate alteration of gene expression by antisense or co-suppression technology or alteration of the functional

properties of the resulting protein molecule. It is therefore understood that the invention encompasses more than the specific exemplary sequences.

For example, it is well known in the art that antisense suppression and co-suppression of gene expression may be accomplished using nucleic acid fragments representing less that the entire coding region of a gene, and by nucleic acid fragments that do not share 100% identity with the gene to be suppressed. Moreover, alterations in a gene which result in the production of a chemically equivalent amino acid at a given site, but do not effect the functional properties of the encoded protein, are well known in the art. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a functionally equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Moreover, the skilled artisan recognizes that substantially similar sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C), with the sequences exemplified herein. Preferred substantially similar nucleic acid fragments of the instant invention are those nucleic acid fragments whose DNA sequences are 80% identical to the DNA sequence of the nucleic acid fragments reported herein. More preferred nucleic acid fragments are 90% identical to the DNA sequence of the nucleic acid fragments reported herein. Most preferred are nucleic acid fragments that are 95% identical to the DNA sequence of the nucleic acid fragments reported herein.

A "substantial portion" of an amino acid or nucleotide sequence comprises enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to afford putative identification of that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) J. Mol. Biol. 215:403-410; see also www.ncbi.nlm.nih.gov/BLAST/). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or

bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of an amino acid or nucleotide sequence comprises enough of the sequence to afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The instant specification teaches partial or complete amino acid and nucleotide sequences encoding one or more particular plant proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

"Codon degeneracy" refers to divergence in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment that encodes all or a substantial portion of the amino acid sequence encoding the *Vernonia galamenensis* fatty acid desaturase enzyme as set forth in SEQ ID NO:2, and to any nucleic acid fragment that encodes all or a substantial portion of the amino acid sequence encoding the *Vernonia galamenensis* fatty acid epoxidizing enzyme as set forth in SEQ ID NO:4. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments which are then enzymatically assembled to construct the entire gene. "Chemically synthesized", as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well established procedures, or automated chemical synthesis can be performed using one of a number of commercially available machines. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell where sequence information is available.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers any gene that is not a native gene,

comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature.

"Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an "enhancer" is a DNA sequence which can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg, (1989) *Biochemistry of Plants 15*:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The "translation leader sequence" refers to a DNA sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G.D. (1995) *Molecular Biotechnology 3*:225).

The "3' non-coding sequences" refer to DNA sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al., (1989) *Plant Cell 1*:671-680.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript; or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA" (mRNA) refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA that is complementary to and derived from mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (U.S. Pat. No. 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that is not translated yet has an effect on cellular processes.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragment of the invention. Expression may also refer to translation of mRNA into a polypeptide. "Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. "Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Pat. No. 5,231,020).

"Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms.

"Mature" protein refers to a post-translationally processed polypeptide; i.e., one from which any pre- or propeptides present in the primary translation product have been removed. "Precursor" protein refers to the primary product of translation of mRNA; i.e., with pre- and propeptides still present. Pre- and propeptides may be but are not limited to intracellular localization signals.

A "chloroplast transit peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the chloroplast or other plastid types present in the cell in which the protein is made. "Chloroplast transit sequence" refers to a nucleotide sequence that encodes a chloroplast transit peptide. A "signal peptide" is an amino acid sequence which is translated in conjunction with a protein and directs the protein to the secretory system (Chrispeels, J.J., (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol. 42*:21-53). If the protein is to be directed to a vacuole, a vacuolar targeting signal (*supra*) can further be added, or if to the endoplasmic reticulum, an endoplasmic reticulum retention signal (*supra*) may be added. If the protein is to be directed to the nucleus, any signal peptide present should be removed and instead a nuclear localization signal included (Raikhel (1992) *Plant Phys.100*:1627-1632).

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms. Examples of methods of plant transformation include Agrobacterium-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol. 143*:277) and particle-accelerated or "gene gun" transformation technology (Klein et al. (1987) *Nature (London) 327*:70-73; U.S. Pat. No. 4,945,050).

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, 1989 (hereinafter "Maniatis").

This invention relates to plant cDNAs with homology to fatty acid desaturase enzymes from other plant species. Several cDNA clones encoding *Vernonia galamenensis* fatty acid modifying enzymes have been isolated and identified by comparison of random plant cDNA sequences to the GenBank database using the BLAST algorithms well known to those skilled in the art. The nucleotide sequence encoding the *Vernonia galamenensis* fatty acid desaturase enzyme is provided in SEQ ID NO:1, and the deduced amino acid sequence is provided in SEQ ID NO:2. The nucleotide sequence encoding the *Vernonia galamenensis* fatty acid epoxidizing enzyme is provided in SEQ ID NO:3, and the deduced amino acid sequence is provided in SEQ ID NO:4. Fatty acid desaturase and epoxidizing enzymes genes from other plants can now be identified by comparison of random cDNA sequences to the *Vernonia galamenensis* sequences provided herein.

The amino acid sequences encoded by the cDNA clones disclosed herein are compared in Figure 1 to the fatty acid desaturase from soybean which inserts the second double bond between carbon atoms 12 and 13 into mono-unsaturated fatty acid, oleic acid, to produce linoleic acid. The sequence of a similar enzyme from castor bean which functions to hydroxylate the number 12 carbon atom of oleic acid to produce ricinoleic acid is also shown for comparison. While both *Vernonia galamenensis* sequences possess the highly conserved amino acid residues that are common to this class of enzyme, one of the clones, vs1.05h08, demonstrates greater similarity to the soybean sequence than vs1.02c07. The amino acid sequence encoded by cDNA clone vs1.05h08 is 70.2% similar to the soybean sequence, while the sequence encoded by cDNA clone vs1.02c07 is only 53.8% similar. As well, the two *Vernonia galamenensis* sequences show only 57.7% similarity to each other as opposed to the much greater sequence similarity shown between species by vs1.05h08.

In *Vernonia galamenensis*, as with other species that produce unusual fatty acids, production of these unusual fatty acids is limited to seed storage tissue. Moreover, the unusual fatty acids are generally not found in other parts of the plant. No signal was detected following Northern analysis of mRNA isolated from leaves of *Vernonia galamenensis* when vs1.02c07 was used as a probe, while the message was very abundant in mRNA isolated from developing seeds.

The sequence relationship of vs1.02c07 to other, known fatty acid desaturases, along with its tissue-specific expression pattern in a tissue that is actively producing the epoxidized fatty acid vernoleate, make it very likely that the enzyme encoded by cDNA clone vs1.02c07 is a fatty acid epoxidase. In contrast, sequence comparisons indicate that cDNA clone vs1.05h08 encodes a fatty acid desaturase enzyme.

The nucleic acid fragments of the instant invention may be used to isolate cDNAs and genes encoding homologous fatty acid modifying enzymes from the same or other plant species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to, methods of nucleic acid hybridization, and methods of DNA and RNA amplification as exemplified by various uses of nucleic acid amplification technologies (e.g., polymerase chain reaction, ligase chain reaction).

For example, genes encoding homologous fatty acid modifying enzymes, either as cDNAs or genomic DNAs, could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired plant employing methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primers DNA labeling, nick translation, or end-labeling techniques, or RNA probes

using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part of or full-length of the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, two short segments of the instant nucleic acid fragments may be used in polymerase chain reaction protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. The polymerase chain reaction may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding plant genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al., (1988) *PNAS USA 85*:8998) to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (BRL), specific 3' or 5' cDNA fragments can be isolated (Ohara et al., (1989) *PNAS USA 86*:5673; Loh et al., (1989) *Science 243*:217). Products generated by the 3' and 5' RACE procedures can be combined to generate full-length cDNAs (Frohman, M.A. and Martin, G.R., (1989) *Techniques 1*:165).

Availability of the instant nucleotide and deduced amino acid sequences facilitates immunological screening cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides can be used to immunize animals to produce polyclonal or monoclonal antibodies with specificity for peptides or proteins comprising the amino acid sequences. These antibodies can be then be used to screen cDNA expression libraries to isolate full-length cDNA clones of interest (Lerner, R.A. (1984) *Adv. Immunol. 36*:1; Maniatis).

The nucleic acid fragments of the instant invention may be used to create transgenic plants in which the *Vernonia galamenensis* fatty acid modifying enzymes are present at higher levels than normal or in cell types or developmental stages in which it is not normally found. For example, when over-expressed in plant cells, the *Vernonia galamenensis* fatty acid epoxidizing enzyme may be useful for causing the biosynthesis and accumulation of epoxidized fatty acids in those cells. It is particularly useful to use the *Vernonia galamenensis* fatty acid epoxidizing enzyme gene to produce epoxidized fatty acids in the cells of the seeds of oilseed crop plants.

Overexpression of the *Vernonia galamenensis* fatty acid epoxidizing or desaturase enzymes may be accomplished by first constructing a chimeric gene in which the *Vernonia*

galamenensis fatty acid epoxidizing or desaturase enzyme coding region is operably linked to a promoter capable of directing expression of a gene in the desired tissues at the desired stage of development. For reasons of convenience, the chimeric gene may comprise a promoter sequence and translation leader sequence derived from the same gene. 3' non-coding sequences encoding transcription termination signals may also be provided. The instant chimeric genes may also comprise one or more introns in order to facilitate gene expression.

Plasmid vectors comprising the instant chimeric genes can then be constructed. The choice of plasmid vector is dependent upon the method that will be used to transform host plants. The skilled artisan is well aware of the genetic elements that must be present on the plasmid vector in order to successfully transform, select and propagate host cells containing the chimeric gene. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., (1985) *EMBO J. 4*:2411-2418; De Almeida et al., (1989) *Mol. Gen. Genetics 218*:78-86), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, Western analysis of protein expression, or phenotypic analysis.

For some applications it may be useful to direct the instant *Vernonia galamenensis* fatty acid modifying enzymes to different cellular compartments, or to facilitate its secretion from the cell. It is thus envisioned that the chimeric genes described above may be further supplemented by altering the coding sequences to encode *Vernonia galamenensis* fatty acid modifying enzymes disclosed herein with appropriate intracellular targeting sequences such as transit sequences (Keegstra, K. (1989) *Cell 56*:247-253), signal sequences or sequences encoding endoplasmic reticulum localization (Chrispeels, J.J., (1991) *Ann. Rev. Plant Phys. Plant Mol. Biol. 42*:21-53), or nuclear localization signals (Raikhel, N. (1992) *Plant Phys.100*:1627-1632) added and/or with targeting sequences that are already present removed. While the references cited give examples of each of these, the list is not exhaustive and more targeting signals of utility may be discovered in the future.

The instant *Vernonia galamenensis* fatty acid modifying enzymes (or portions of the enzymes) may be produced in heterologous host cells, particularly in the cells of microbial hosts, and can be used to prepare antibodies to the *Vernonia galamenensis* fatty acid epoxidizing and desaturase enzymes by methods well known to those skilled in the art. The antibodies are useful for detecting the instant *Vernonia galamenensis* fatty acid modifying enzymes *in situ* in cells or *in vitro* in cell extracts. Preferred heterologous host cells for production of the instant *Vernonia galamenensis* fatty acid modifying enzymes are microbial hosts. Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art.

Any of these could be used to construct chimeric genes for production of the instant *Vernonia galamenensis* fatty acid epoxidizing enzyme or the instant *Vernonia galamenensis* fatty acid desaturase enzyme. These chimeric genes could then be introduced into appropriate microorganisms via transformation to provide high level expression of the encoded *Vernonia galamenensis* fatty acid modifying enzyme. An example of a vector for high level expression of the instant *Vernonia galamenensis* fatty acid modifying enzymes in a bacterial host is provided (Example 8).

All or a portion of the nucleic acid fragments of the instant invention may also be used as probes for genetically and physically mapping the genes that they are a part of, and as markers for traits linked to expression of the instant fatty acid epoxidizing and desaturase enzymes. Such information may be useful in plant breeding in order to develop lines with desired phenotypes.

For example, the instant nucleic acid fragments may be used as restriction fragment length polymorphism (RFLP) markers. Southern blots (Maniatis) of restriction-digested plant genomic DNA may be probed with the nucleic acid fragments of the instant invention. The resulting banding patterns may then be subjected to genetic analyses using computer programs such as MapMaker (Lander et at., (1987) *Genomics 1*:174-181) in order to construct a genetic map. In addition, the nucleic acid fragments of the instant invention may be used to probe Southern blots containing restriction endonuclease-treated genomic DNAs of a set of individuals representing parent and progeny of a defined genetic cross. Segregation of the DNA polymorphisms is noted and used to calculate the position of the instant nucleic acid sequence in the genetic map previously obtained using this population (Botstein, D. et al., (1980) *Am.J.Hum.Genet.32*:314-331).

The production and use of plant gene-derived probes for use in genetic mapping is described in R.Bernatzky, R. and Tanksley, S.D. (1986) *Plant Mol.Biol.Reporter 4(1)*:37-41. Numerous publications describe genetic mapping of specific cDNA clones using the methodology outlined above or variations thereof. For example, F2 intercross populations, backcross populations, randomly mated populations, near isogenic lines, and other sets of individuals may be used for mapping. Such methodologies are well known to those skilled in the art.

Nucleic acid probes derived from the instant nucleic acid sequences may also be used for physical mapping (i.e., placement of sequences on physical maps; *see* Hoheisel, J. D., et al., In: *Nonmammalian Genomic Analysis: A Practical Guide*, Academic press 1996, pp. 319-346, and references cited therein).

In another embodiment, nucleic acid probes derived from the instant nucleic acid sequences may be used in direct fluorescence *in situ* hybridization (FISH) mapping. Although current methods of FISH mapping favor use of large clones (several to several

hundred KB), improvements in sensitivity may allow performance of FISH mapping using shorter probes.

A variety of nucleic acid amplification-based methods of genetic and physical mapping may be carried out using the instant nucleic acid sequences. Examples include allele-specific amplification, polymorphism of PCR-amplified fragments (CAPS), allele-specific ligation, nucleotide extension reactions, Radiation Hybrid Mapping and Happy Mapping. For these methods, the sequence of a nucleic acid fragment is used to design and produce primer pairs for use in the amplification reaction or in primer extension reactions. The design of such primers is well known to those skilled in the art. In methods employing PCR-based genetic mapping, it may be necessary to identify DNA sequence differences between the parents of the mapping cross in the region corresponding to the instant nucleic acid sequence. This, however, is generally not necessary for mapping methods. Such information may be useful in plant breeding in order to develop lines with desired starch phenotypes.

EXAMPLES

The present invention is further defined in the following Examples, in which all parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

EXAMPLE 1

Composition of a cDNA Library; Isolation and Sequencing of cDNA Clones

A cDNA library representing mRNAs from developing seeds of *Vernonia galamenensis* that had just begun production of vernolic acid was prepared. The library was prepared in a Uni-ZAP™ XR vector according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). Conversion of the Uni-ZAP™ XR library into a plasmid library was accomplished according to the protocol provided by Stratagene. Upon conversion, cDNA inserts were contained in the plasmid vector pBluescript. cDNA inserts from randomly picked bacterial colonies containing recombinant pBluescript plasmids were amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Amplified insert DNAs were sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams, M. D. et al., (1991) *Science 252*:1651). The resulting ESTs were analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

EXAMPLE 2

Identification and Characterization of cDNA Clones

ESTs encoding *Vernonia galamenensis* fatty acid modifying enzymes were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) J. Mol. Biol. 215:403-410; see also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish, W. and States, D. J. (1993) *Nature Genetics 3*:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

The BLASTX search using the nucleotide sequence from clone vs1.05h08 revealed similarity of the protein encoded by the cDNA to, *inter alia*, a tomato fatty acid desaturase enzyme (EMBL Accession No. X94944; pLog = 1.72) and a potato fatty acid desaturase enzyme (EMBL Accession No. X92847; pLog = 0.52). The sequence of the entire cDNA insert in clone vs1.05h08 was determined and reevaluated by BLAST, yielding even higher pLog values vs. the potato fatty acid desaturase enzyme (X92847; pLog = 228.04). SEQ ID NO:1 shows the nucleotide sequence of the entire *Vernonia galamenensis* cDNA in clone vs1.05h08; the deduced amino acid sequence is shown in SEQ ID NO:2. Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragment encodes a *Vernonia galamenensis* fatty acid desaturase.

The BLASTX search using the nucleotide sequence of clone vs1.02c07 revealed similarity of the protein encoded by the cDNA to, *inter alia*, a potato fatty acid desaturase enzyme (EMBL Accession No. X92847; pLog = 2.08) and a soybean fatty acid desaturase enzyme (GenBank Accession No. L43921; pLog = 1.49). The sequence of the entire cDNA insert in clone vs1.02c07 was determined and reevaluated by BLAST, yielding even higher pLog values vs. the potato (X92847; pLog = 156.18) and soybean fatty acid desaturase enzymes (L43921; pLog = 152.18). SEQ ID NO:3 shows the nucleotide sequence of the

entire *Vernonia galamenensis* cDNA; the deduced amino acid sequence is shown in SEQ ID NO:4.

The deduced amino acid sequences from cDNA clones vs1.05h08 and vs1.02c07 were compared to the deduced amino acid sequences encoding (i) a know fatty acid desaturase from soybean (World Patent Publication No. WO94/11516) and (ii) a fatty acid hydroxylase from castor bean (van de Loo, F. J. et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92 (15)*:6743-6747) using the multiple sequence comparison program "Megalign" from the Lasargene™ software package (DNASTAR Inc., Madison, WI). The aligned sequences are shown in Figure 1. All four sequences are related by eight very highly conserved residues that are apparently part of the binding site for the two iron cluster that is required in the active site of enzymes in this class (Shanklin, J. et al. (1994) *Biochemistry 33*:12787-12793). The cDNA insert in clone vs1.05h08 is about 70% similar to the known fatty acid desaturase from soybean, while the cDNA insert in vs1.02c07 is only 53.8% similar to this soybean fatty acid desaturase. This degree of divergence is similar to that observed between the fatty acid hydroxlyase from castor bean and the fatty acid desaturases. Thus, changes in a comparatively small number of amino acid residues in conserved regions of the protein are sufficient to alter the activity in this class of enzymes from one of introducing a double bond (i.e, a desaturase) to one of introducing an hydroxyl group (i.e., a hydroxylase).

The sequence of clone vs1.02c07 is also quite divergent from the castor bean fatty acid hydroxylase, sharing only 52.5% similar residues. The sequence of vs1.02c07 is therefore unique, but nonetheless related, to the class of enzymes which is known to contain enzymes capable of producing different modifications in the acyl chains of fatty acids. Sequence alignments, BLAST scores and probabilities and experimental data demonstrating a tissue-specific expression pattern (see Example 3) for the gene encoded by the cDNA clone vs1.02c07 indicate that the instant nucleic acid fragment comprising this clone encodes a *Vernonia galamenensis* fatty acid epoxidizing enzyme.

EXAMPLE 3

Tissue Specific Expression of the Fatty Acid Epoxidase

Expression of enzymes which produce unusual fatty acids has been shown to be specific for the storage organs in which these fatty acids are found (see World Patent Publication No. WO94/11516). Northern analysis of mRNA from developing leaves of *Vernonia galamenensis* was performed using the cDNA insert in vs1.02c07 as a probe. Messenger RNA from leaves that were near full expansion (by comparison to other leaves just lower on the main stem) were removed and mRNA was prepared by standard methods well known in the art. The leaf mRNA, and a remaining sample of the developing seed mRNA that was used as template for the cDNA library from which the instant cDNA clones were obtained, was separated by denaturing agarose gel electrophoresis and blotted to a nylon membrane for hybridization to the probe.

The ^{32}P-labeled probe based on the cDNA insert in vs1.02c07 was prepared by PCR amplification of the coding region of the cDNA using the nucleotide described in SEQ ID NO:7 as the 5' end primer and the nucleotide described in SEQ ID NO:8 as the 3' end primer. The product from PCR amplification was purified by isolation from an agarose gel and used as template for random primed labeling. The portion of cDNA clone vs1.05h08 encoding the peptide was similarly amplified using the nucleotides described in SEQ ID NOs:9 and 10 as the 5' and 3' primers, respectively, and the purified product used as probe template.

Both probes were hybridized to the Northern blots at 62°C in 0.2X SSC overnight. Excess probe was removed by washing under the same stringency conditions and the blot was placed on photographic film for development.

The lane containing the seed mRNA produced an intense hybridization signal at about 1.9kD when the coding region of vs1.02c07 was used as the probe, while no signal was visible in the lane containing the leaf-derived mRNA. Further exposure of the blot, such that the seed-derived signal was highly over exposed, still did not result in a visible signal in the lane containing the leaf-derived message. The coding region of vs1.05h08 hybridized to message from both developing seeds and from leaves.

The cDNA insert in vs1.02c07 is therefore not expressed in a *Vernonia galamenensis* tissue that does not produce epoxidized fatty acids, but is highly expressed in a tissue that does produce the modified fatty acid. The tissue-specific nature of its expression, its relationship to a known class of fatty acid modifying enzymes, and its divergence from enzymes in that class whose catalytic function has been demonstrated, all indicate that the cDNA insert in vs1.02c07 encodes the fatty acid epoxidizing enzyme from *Vernonia galamenensis*.

EXAMPLE 4

Expression of Chimeric Genes in Monocot Cells

The oil storing tissues of most grass seeds are the embryo and its attending tissues the scutellum and to some extent the aleurone. Promoter sequences such as those controlling expression of the storage proteins Globulin 1 (Belanger, S.C. and Kriz, A. L. (1989) *Plant Physiol. 91*:636-643) and Globulin 2 (Wallace, N.H. and Kriz, A.L. (1991) *Plant Physiol. 95*:973-975) are appropriate for the expression of chimeric genes in these tissues..

A chimeric gene comprising a cDNA encoding a *Vernonia galamenensis* fatty acid epoxidizing enzyme in sense orientation with respect to the maize Globulin 2 promoter that is located 5' to the cDNA fragment, and the Globulin 2 3' end that is located 3' to the cDNA fragment, can be constructed. The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the correctly designed expression vector.

Such expression vectors should include genetic sequence elements conferring an origin of replication for the plasmid in its host, a gene capable of conferring a selectable trait such as autotrophy or antibiotic tolerance to the host cell carrying the plasmid, and the promoter sequences for expression of desired genes in host plant cells. Further design features may include unique restriction endonuclease recognition sites between the elements of the plant gene promoter elements to allow convenient introduction genes to be controlled by those elements.

The chimeric genes constructed as above can then be introduced into corn cells by the following procedure. Immature corn embryos can be dissected from developing caryopses derived from crosses of the inbred corn lines H99 and LH132. The embryos are isolated 10 to 11 days after pollination when they are 1.0 to 1.5 mm long. The embryos are then placed with the axis-side facing down and in contact with agarose-solidified N6 medium (Chu et al., (1975) *Sci. Sin. Peking* 18:659-668). The embryos are kept in the dark at 27°C. Friable embryogenic callus consisting of undifferentiated masses of cells with somatic proembryoids and embryoids borne on suspensor structures proliferates from the scutellum of these immature embryos. The embryogenic callus isolated from the primary explant can be cultured on N6 medium and sub-cultured on this medium every 2 to 3 weeks.

The plasmid, p35S/Ac (obtained from Dr. Peter Eckes, Hoechst Ag, Frankfurt, Germany) may be used in transformation experiments in order to provide for a selectable marker. This plasmid contains the *Pat* gene (see European Patent Publication 0 242 236) which encodes phosphinothricin acetyl transferase (PAT). The enzyme PAT confers resistance to herbicidal glutamine synthetase inhibitors such as phosphinothricin. The *pat* gene in p35S/Ac is under the control of the 35S promoter from Cauliflower Mosaic Virus (Odell et al. (1985) *Nature* 313:810-812) and the 3' region of the nopaline synthase gene from the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*.

The particle bombardment method (Klein et al., (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold particles (1 μm in diameter) are coated with DNA using the following technique. Ten μg of plasmid DNAs are added to 50 μL of a suspension of gold particles (60 mg per mL). Calcium chloride (50 μL of a 2.5 M solution) and spermidine free base (20 μL of a 1.0 M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200 μL of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30 μL of ethanol. An aliquot (5 μL) of the DNA-coated gold particles can be placed in the center of a Kapton™ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a Biolistic™ PDS-1000/He (Bio-Rad

Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

For bombardment, the embryogenic tissue is placed on filter paper over agarose-solidified N6 medium. The tissue is arranged as a thin lawn and covered a circular area of about 5 cm in diameter. The petri dish containing the tissue can be placed in the chamber of the PDS-1000/He approximately 8 cm from the stopping screen. The air in the chamber is then evacuated to a vacuum of 28 inches of Hg. The macrocarrier is accelerated with a helium shock wave using a rupture membrane that bursts when the He pressure in the shock tube reaches 1000 psi.

Seven days after bombardment the tissue can be transferred to N6 medium that contains gluphosinate (2 mg per liter) and lacks casein or proline. The tissue continues to grow slowly on this medium. After an additional 2 weeks the tissue can be transferred to fresh N6 medium containing gluphosinate. After 6 weeks, areas of about 1 cm in diameter of actively growing callus can be identified on some of the plates containing the glufosinate-supplemented medium. These calli may continue to grow when sub-cultured on the selective medium.

Plants can be regenerated from the transgenic callus by first transferring clusters of tissue to N6 medium supplemented with 0.2 mg per liter of 2,4-D. After two weeks the tissue can be transferred to regeneration medium (Fromm et al., (1990) *Bio/Technology 8*:833-839).

EXAMPLE 5

Expression of Chimeric Genes in Dicot Cells

The *Vernonia galamenensis* fatty acid epoxidizing enzyme was expressed in cells of two dicots that normally produce storage lipid by the construction of appropriate chimeric genes followed by stable introduction of those genes into the host plants.

Soybean embryo expression

A plasmid designated pKS18HH containing chimeric genes to afford expression of Hygromycin B Phosphotransferase in certain bacteria and in plant cells was constructed from the following genetic elements: a) T7 Promoter and Shine-Delgarno sequence/Hygromycin B Phosphotransferase (HPT)/T7 Terminator Sequence, b) 35S Promoter from cauliflower mosaic virus (CaMV)/Hygromycin B Phosphotransferase (HPT)/Nopaline Synthase (NOS 3' from *Agrobacterium tumefaciens* T-DNA, and c) pSP72 plasmid vector (Promega Biotech) with beta-lactamase coding region (ampicillin resistance gene) removed. The Hygromycin B Phosphotransferase gene was obtained by PCR amplification of the *Klebsiella* derived plasmid pJR225 (Gritz, L. and Davies, J. (1983) *Gene 25(2-3)*:179-88) in *E. coli* strain W677. Starting with the pSP72 vector, the elements were assembled into a single plasmid using standard cloning methods (Maniatis).

Plasmid pKS18HH thus contains the T7 promoter/HPT/T7 terminator cassette for expression of the HPT enzyme in certain strains of *E. coli*, such as NovaBlue(DE3) (Novagen), that are lysogenic for lambda DE3 (which carries the T7 RNA Polymerase gene under lacUV5 control). Plasmid pKS18HH also contains the 35S/HPT/NOS cassette for constitutive expression of the HPT enzyme in plants, such as soybean. These two expression systems allow selection for growth in the presence of hygromycin to be used as a means of identifying cells that contain the plasmid in both bacterial and plant systems. Plasmid pKS18HH also contains three unique restiction endonuclease sites suitable for the cloning other chimeric genes into this vector.

A plasmid for expression of the cDNA encoding the *Vernonia galamenensis* fatty acid epoxidizing enzyme under control of the soybean beta-conglycinin promoter (Beachy et al. (1985) *EMBO J. 4*:3047-3053) was constructed. The construction of this vector was facilitated by the use of plasmids pCW109 and pML18, both of which have been described (see World Patent Publication No. WO94/11516).

A unique Not I site was introduced into the cloning region between the beta-conglicinin promoter and the phaseolin 3' end in pCW109 by digestion with Nco I and Xba I followed by removal of the single stranded DNA ends with mung bean exonuclease. Not I linkers (New England Biolabs Inc., cat. No. NEB 1125) were ligated into the linearized plasmid to produce plasmid pAW35. The single Not I site in pML18 was destroyed by digestion with Not I, filling in the single stranded ends with dNTP's and Klenow fragment followed by re-ligation of the linearized plasmid. The modified pML18 was then digested with Hind III and treated with calf intestinal phosphatase.

The beta-conglicinin/Not I/phaseolin expression cassette in pAW35 was removed by digestion with Hind III and the 1.79 kB fragment was isolated by agarose gel electrophoresis. The isolated fragment was ligated into the modified and linearized pML18 construction described above. A clone with the desired orientation was identified by digestion with Not I and Xba I to release a 1.08 kB fragment indicating that the orientation of the beta-conglycinin transcription unit was the same as the selectable marker transcription unit. The resulting plasmid was given the name pBS19.

Hind III is one of the unique cloning sites available in pKS18HH. To assemble the final expression cassette, pBS19 and pKS18HH were both digested with Hind III. The beta-conglycinin containing fragment from pBS19 was isolated by gel electrophoresis and ligated into the digested pKS18HH which had been treated with calf alkaline phosphatase. The resulting plasmid was named pRB20.

The PCR product amplified from clone vs1.02c07 (described in Example 3 above) was digested with Not I to cleave the Not I sites designed into the PCR primers. Plasmid pRB20 was also Not I digested. After phosphatase treatment of the linearized pRB20, the Not I digested vs1.02c07 product was ligated into pRB20 and the ligation mixture used to

transform *E. coli* strain DE3. Colonies were selected and grown in liquid media for preparation of plasmid DNA. Digestion of the plasmid DNA with Xmn I released a fragment of 0.4 kB when the coding sequence of vs1.02c07 was oriented in the sense direction relative to the beta-conglycinin promoter. The selected clone was designated pRVF7 and the restriction endonuclease map of the plasmid is shown in Figure 2. The *E. coli* cell line containing pRVF7 was grown and plasmid DNA isolated to produce DNA for stable transformation of soybean.

Soybean embryos were transformed with the expression vector pRFV7. Media designations and recipes are given in Table 1 below. To induce somatic embryos, cotyledons 3-5 mm in length dissected from surface sterilized, immature seeds of the soybean cultivar A2872, were cultured in the light or dark at 26°C on agar medium SB1 for 6-10 weeks. Somatic embryos which produced secondary embryos were excised and placed into liquid medium SB 172. After repeated selection for clusters of somatic embryos which multiplied as early, globular staged embryos, the suspensions were maintained as described below.

Soybean embryogenic suspension cultures were maintained in 35 mL of SB 172 media on a rotary shaker, 150 rpm, at 26°C with florescent lights on a 16:8 hour day/night schedule. Cultures were subcultured every two weeks by inoculating approximately 35 mg of tissue into 35 mL of SB 172 medium.

Soybean embryogenic suspension cultures were transformed by the method of particle gun bombardment (Kline et al. (1987) *Nature (London) 327*:70; U.S. Patent No. 4,945,050) using a Du Pont Biolistic™ PDS1000/HE instrument (helium retrofit) can.

To 50 mL of a 60 mg/mL 1 mm gold particle suspension was added (in order): 5 mL of plasmid pRVF7 DNA (1 mg/mL), 20 ml spermidine (0.1 M), and 50 mL CaCl2 (2.5 M). The particle preparation was agitated for three minutes, spun in a microfuge for 10 seconds and the supernatant removed. The DNA-coated particles were washed once in 400 mL 70% ethanol and resuspended in 40 mL of anhydrous ethanol. The DNA/particle suspension was sonicated three times for one second each. Five mL of the DNA-coated gold particles were then loaded on each macro carrier disk.

Approximately 300-400 mg of a two-week-old suspension culture was placed in an empty 60x15 mm petri dish and the residual liquid removed from the tissue with a pipette. For each transformation experiment, approximately 5-10 plates of tissue were bombarded. Membrane rupture pressure was set at 1100 psi and the chamber was evacuated to a vacuum of 28 inches mercury. The tissue was placed approximately 3.5 inches away from the retaining screen and bombarded three times. Following bombardment, the tissue was divided in half and placed back into liquid and cultured as described above.

Three to four days post bombardment, the liquid media was exchanged with fresh media, and six to eleven days post bombardment with fresh SB 172 media containing

50 mg/L hygromycin. This selective media was refreshed weekly. Four to six weeks post bombardment, green, transformed tissue was observed growing from untransformed, necrotic embryogenic clusters. Isolated green tissue was removed and inoculated into individual cells of six cell well plates. Each well contained six ml of SB 172 media with no hygromycin. Cultures were maintained for two to six weeks with media change every two weeks. Surviving cultures are treated as independent transformation events and were returned to 35 mL SB 172 with hygromycin in culture flasks for tissue increase for four to six weeks with media changes every two weeks.

Embryonic clusters were removed from liquid culture and placed on solid agar SB166 media for one week then subcultured to SB103 for 3 weeks. Individual, maturing embryos were removed for fatty acid analysis as described in Example 7 at this point.

To regenerate fertile soybean plants, embryos from selected lines were desiccated by placing embryos into a small, empty petri plate that was placed inside a sealed, larger petri plate containing SB103 media. Partially desiccated embryos that were yellow in color were returned to petri plates containing either SB71-3 or SB71-4 media and allowed to germinate. Embryos that germinated and produced roots and leaves were transferred to sterile soil and grown to maturity for seed collection.

Table 1. Transformation Media

SB172 (per liter)	1 bottle Duchefa salts (MS medium-Finer modification)* 1 ml B5 vitamins 1 ml 2,4-D stock 60 g sucrose 0.667 g asparagine pH 5.7
SB1 (per liter)	1 pkg MS salts (Gibco/BRL - premade formulation) 1 ml B5 vitamins 60 g sucrose 4 ml 2,4-D stock pH 5.8 0.8% agar
SB166 (per liter)	1 pkg MS salts (Gibco/BRL - premade formulation) 1 ml B5 vitamins 60 g maltose 750 mg $MgCl_2$ hexahydrate 5 g activated charcoal pH 5.7 2 g gelrite

SB103 (per liter)	1 pkg MS salts (Gibco/BRL - premade formulation 1 ml B5 vitamins 60 g maltose 750 mg $MgCl_2$ hexahydrate pH 5.7 2 g gelrite
SB 148 (per liter)	1 pkg MS salts (Gibco/BRL - premade formulation) 1 ml B5 vitamins 60 g maltose pH 5.7 7 g agarose
SB 71-3 (per liter)	1 bottle B5 salts w/ sucrose (Gibso/BRL - premade formulation) 750 mg $MgCl_2$ hexahydrate pH 5.7 5 g TC agar
SB 71-4 (per liter)	1 bottle B5 salts w/ sucrose (Gibco/BRL - premade formulation) pH 5.7 5 g TC agar
2,4-D stock	10 mg/ml
B5 vitamins	1000X stock - (per 100 ml) 10 g myo-inositol 100 mg nicotinic acid 100 mg pyridoxine HCl 1 g thiamine

*Duchefa Biochemie B.V., Izaak Enschedeweg 40, NL-2031 CS Haarlem, Netherland

EXAMPLE 6

Seed Specific Expression of the Vernonia Fatty Acid Epoxidase in *Arabidopsis thaliana*

Arabidopsis thaliana was transformed with a vector designed to allow seed specific expression of the vernonia sequence vs1.02c07 using the *in planta* transformation proceedure described by Bechtold et al. ((1993) *CR Acad Sci Paris 316*:1194-1199). The expression vector was constructed using two existing plasmids. Plasmid pML2 contained the cDNA sequence for a mutant form of the enzyme acetolactate synthase from *Arabidopsis* under the control of the 35S promoter. Transfer to plants confers resistance to the sulfonyl urea herbicide chlorsufuron. Plasmid pIMC01 contains the 5' and 3' regulatory regions surrounding the *Brassica* storage protein Napin with added restriction endonuclease sites between the 5' and 3' regulatory regions. Its construction is described in WO94/11516 as is

the Agrobacterium binary vector pZS94K. NcoI and SalI sites were introduced on the 5' and 3' ends of vs1.02c07 by PCR using primers that contain the recognition sequences for those enzymes 5' and 3' respectively to the target sequences at the ends of vs1.02c07. The PCR product was digested with NcoI and SalI and ligated into pIMC01 that had also been digested with NcoI and SalI.

Seed from the primary transformants was planted in flats, cold treated before gerimation and the seedlings were sprayed with 50 mg/L chlorsulfuron. Surviving seedlings were transplanted, taken to maturity and seeds were analyzed for the presence of the 12, 13 epoxide containing vernolic acid as described below. Seeds from two lines that produced seeds segregating for production of vernolic acid were planted and the seedlings were sprayed with 200 mg/L chlorsulfuron. The surviving seedlings were again grown to maturity and the resulting seeds analyzed for the presence of vernolic acid.

EXAMPLE 7

Analysis of Vernolic Acid in Transgenic Plant Tissues

The *Arabidopsis* transformation proceedure resulted in many apparent escape events as only one in twenty plants chosen as possible transformants had a seed fatty acid profile that included the putative vernolic acid. That one event accumulted the new fatty acid to about 1% of the total fatty acids in individual seeds from the transformant. This accumulation level was sufficient to allow positive identificationof the putative vernolic acid derivative by GC-mass spectrometry. GC-MS was done first on the total fatty acids from a single seed which was shown by GC analysis to contain the putative vernolic methyl ester by the method described in Hitz et al. ((1994) *Plant Physiol 105*:635-641). The mass spectra of the major peak in the methyl esters obtained from mature *Vernonina galemenensis* had a weak molecular ion at M/e+ 310 and a slightly more prominent signal at (M-31) of 279. This is a common inital loss of the OCH_3 from fatty acid methyl esters. While the transgenic arabidopsis seed did not provide sufficient mass spectral signal to identify the molecular ion in the putative vernolic acid methyl ester peak the signal at M/e+ 279 was present and the remainder of the spectra was identical to the main peak from vernonia.

Single seed analysis of twelve seeds from one of the *Arabidopsis* plants selected by spraying with chlorsulfuron in the second generation after transformation all contained the putative vernolic acid at about 1.5% of the total fatty acids. The plant is apparently homozygous for the transgene.

Methyl esterification of the fatty acids from *Vernonia* under acid conditions results in a slow methanolysis of the 12, 13 epoxide to a mixture of the 12-OH, 13- OCH_3 and the 12-OCH_3, 13-OH methyl esters. Bulk seed from the selected, homozygous transformant was treated with 1% H_2SO_4 in methanol at reflux for 4 h and the resulting fatty acid methyl esters run on GC-MS as above. The larger of the two peaks that were unique to the transformed arabidopsis fatty acids had a spectra characteristic of the 12-OH, 13-OCH3

methyl ester derivative of the methanolysis product of vernolic acid: a prominent fragment at M+/e of 227 corresponding to the portion of the molecule from the carboxyl end to the 12 carbon and the base ion at M+/e 115 corresponding to the fragment from carbons 13 through 18. The other peak produced a spectra characteristic of the 13- OCH_3, 12-OH methyl ester: the M+/e fragment at 241 coresponding to the split between carbons 12 and 13 with the 12 carbon carrying the methoxy group and the base ion at M+/e 145 from the split between carbons 11 and 12.

The results from the *Arabadopsis* transformations prove that the enzyme product encoded by SEQ ID NO:3 is capable of catalyzing the production of vernolic acid when transgenically introduced into a plant that does not normally produce or accumulate vernolic acid.

Fatty acids in somatic soybean embryos were directly transesterified in methanol containing 1% sodium methoxide. The fatty acid methyl esters were extracted into heptane and a portion of the heptane extract was seperated by gas liquid chromatography as described in Hitz et al. ((1994) *Plant Physiol 105*:635-641). Ten soybean embryos from each of 8 transgenic lines were assayed individually and the results are given in Table 2.

Table 2. Individual fatty acids as a per cent of total fatty acid in single somatic embryos obtained after transformation with plasmid pRFV7.

Line#	Sample	16:0	18:0	18:1	18:2	18:3	Vern
2065/3/6/1	2065361.0	14.5	2.5	8.8	58.7	15.1	0.4
	2065361.1	12.9	3.1	13.9	56.3	12.3	1.5
	2065361.2	15.2	3.7	12.2	51.7	16.2	1.1
	2065361.3	0.0	4.5	18.2	55.3	19.0	2.9
	2065361.4	17.0	4.3	9.4	49.3	18.2	1.8
	2065361.5	0.0	6.1	14.5	59.0	19.5	0.9
	2065361.6	16.2	5.3	12.9	48.4	15.3	1.9
	2065361.7	14.9	4.9	10.0	51.3	15.5	3.5
	2065361.8	14.0	3.6	12.8	55.1	13.3	1.3
	2065361.9	16.6	4.5	11.5	50.0	15.5	1.8
2065/3/6/3	2065363.0	13.8	4.1	13.8	34.1	28.5	5.8
	2065363.1	13.7	3.3	11.7	50.1	20.1	1.1
	2065363.2	16.1	4.3	10.1	39.2	25.9	4.4
	2065363.3	13.7	4.5	11.0	50.3	18.5	2.0
	2065363.4	11.8	3.7	14.4	54.0	14.4	1.7
	2065363.5	18.7	4.9	8.0	40.6	27.8	0.0
	2065363.6	13.0	2.6	10.1	54.2	17.9	2.3
	2065363.7	13.0	3.1	11.2	55.7	16.1	0.9
	2065363.8	19.5	4.3	7.9	43.9	24.4	0.0
	2065363.9	16.0	3.5	7.5	49.1	23.3	0.7

Line#	Sample	16:0	18:0	18:1	18:2	18:3	Vern
2065/3/11/1	20653111.0	15.3	2.5	8.3	53.6	20.2	0.0
	20653111.1	15.6	5.4	10.3	49.3	19.1	0.3
	20653111.2	15.9	3.5	10.0	46.7	21.4	2.5
	20653111.3	12.3	2.7	14.7	57.8	12.5	0.0
	20653111.4	16.6	4.4	8.2	51.3	18.0	1.5
	20653111.5	14.8	3.2	8.5	51.2	20.3	2.0
	20653111.6	13.9	2.9	11.0	58.4	13.9	0.0
	20653111.7	12.9	3.4	9.6	58.5	15.6	0.0
	20653111.8	14.4	3.9	6.5	54.0	19.2	2.0
	20653111.9	13.6	3.0	11.6	58.3	13.5	0.0
2065/3/11/2	20653112.0	12.8	3.6	11.4	53.8	18.2	0.0
	20653112.1	11.0	2.8	15.4	55.5	15.3	0.0
	20653112.2	16.3	4.6	10.8	45.8	20.0	2.6
	20653112.3	12.3	3.3	11.3	52.5	19.6	1.1
	20653112.4	15.8	4.0	6.6	34.1	30.5	8.9
	20653112.5	17.3	4.5	9.2	35.9	28.4	4.8
	20653112.6	15.5	3.4	9.8	40.5	27.4	3.4
	20653112.7	16.3	4.1	8.2	37.4	28.1	6.0
	20653112.8	17.7	4.8	8.4	34.7	27.9	6.4
	20653112.9	17.6	3.7	6.2	36.6	27.7	8.3
2092/1/1	2092-1-1-1	12.8	4.6	14.2	54.7	13.1	0.0
22-Oct	-2.0	11.5	3.7	13.0	55.9	15.4	0.0
	-3.0	11.4	3.4	11.8	59.8	13.2	0.0
	-4.0	17.9	3.8	3.2	32.7	41.3	0.0
	-5.0	13.2	3.3	11.3	56.2	15.5	0.0
	-6.0	13.1	3.0	11.3	58.1	14.1	0.0
	-7.0	16.9	3.5	5.6	48.7	24.5	0.0
	-8.0	14.0	3.7	7.1	57.9	16.6	0.0
	-9.0	14.1	3.2	7.5	49.8	24.6	0.3
	-10.0	16.8	3.6	4.9	43.0	30.6	0.0
2092/2/1	2092-2-1-1	18.1	4.2	7.4	38.6	30.1	0.8
	-2.0	17.4	3.8	3.4	46.9	27.4	0.0
	-3.0	13.0	3.4	12.6	57.3	13.0	0.1
	-4.0	12.7	3.6	13.3	56.8	13.0	0.1
	-5.0	14.0	4.5	9.4	50.9	19.9	0.3
	-6.0	13.4	2.8	11.8	59.3	11.7	0.5
	-7.0	15.8	3.3	8.8	55.2	15.9	0.2
	-8.0	13.8	3.5	10.7	56.0	15.5	0.0
	-9.0	18.8	3.9	8.7	43.1	24.8	0.0
	-10.0	14.1	2.7	10.2	52.1	19.6	0.6

Line#	Sample	16:0	18:0	18:1	18:2	18:3	Vern
2092/2/2	2092-2-2-1	13.0	5.0	11.7	54.6	14.9	0.0
	-2.0	16.2	4.4	4.3	56.5	18.1	0.0
	-3.0	15.1	3.5	8.4	48.9	23.3	0.0
	-4.0	15.5	4.1	5.3	56.0	18.5	0.0
	-5.0	21.6	5.4	8.4	33.7	30.1	0.0
	-6.0	15.6	5.1	9.2	50.1	19.2	0.0
	-7.0	15.4	5.1	8.2	52.0	18.6	0.0
	-8.0	14.0	5.0	9.4	55.6	15.1	0.0
	-9.0	13.7	4.3	9.7	57.6	14.2	0.0
	-10.0	12.4	3.5	9.1	58.0	16.4	0.0
2092/3/1	2092-3-1-1	13.5	3.2	11.2	57.0	14.5	0.0
	-2.0	15.6	3.8	7.2	57.4	15.2	0.0
	-3.0	12.9	2.9	12.4	57.1	14.0	0.0
	-4.0	12.8	2.5	9.9	60.4	13.9	0.0
	-5.0	16.6	3.9	6.8	52.2	19.7	0.0
	-6.0	0.0	5.0	4.1	89.9	0.0	0.0
	-7.0	13.3	3.7	7.5	55.1	19.7	0.0
	-8.0	12.6	4.7	14.7	55.1	12.2	0.0
	-9.0	12.1	3.3	13.4	57.4	13.4	0.0
	-10.0	16.4	3.9	8.9	43.1	26.0	0.0

A fatty acid idnetified by retention time as vernolic acid methyl ester was found in five of the eight lines. The amount of the fatty acid varied from one transformation event to another and to a lesser extent between embryos within an event. The maximum amount of vernolic acid accumulated was about 8% of the total fatty acids.

Accumulation of Vernolic Acid in Soybean Seeds

A total of 20 plants were recovered from the three transformation events from bombardment number 2065 described above, 14 plants were recovered from bombardment 2092, and a further 10 plants were recovered from bombardment 798 that was not analyzed at the somatic embryo stage.

Since seeds from the primary transformant are genetically segregating for the transgene in the first generation after transformation, single seeds from each plant were analyzed to estimate the segregation ratio and the possible phenotype of the homozygous seed. A small piece of cotyledon tissue was cut from each of 15 single seeds from each plant. The fatty acids in the sample were derivatized and the fatty acid profile determined by GLC as described above. If care is taken to avoid the seed embryo during sampling the remaining seed can be germinated. This seed ship method accurately predicts the fatty acid profile of the whole seed and allows recovery of a plant from a seed of known phenotype.

Only three of the plants recovered from bombardment 2092 accumulated any vernolic acid and those accumulated less than 1% of the total seed fatty acids as vernolic. Sixteen of the 20 plants recovered from bombardment 2065 were accumulating the epoxy fatty acid in the segregating seeds. The maximum accumulation was about 9% of total fatty acids. All

ten plants from bombardment 798 accumulated vernolic acid and a total of 165 single seeds were analyzed by the seed chip method to recover putative homozygotes for advancement of the population. The results are shown as a class histogram in Figure 3. The number of seeds which were did not accumulate a significant amount of vernolic acid suggests that the phenotype follows a single gene segregation pattern. If that is the case then the population of seeds comprising the upper 25% in terms of accumulation should contain the seeds homozygous for the transgene. That group is quite broad in this population but centers on about 7% vernolic acid (Figure 3).

Seeds from within the upper 25% of accumulators and from the population of non-accumulators were planted. Leaf tissue for DNA extraction was taken from vegetative plants from the population derived from seeds that were accumulating vernolic acid and those that were not. Southern analysis revealed a single hybridizing band of about 3 kB in size from DNA digested with Hind III in DNA from plants derived from accumulting seeds but no corresponding band from non-accumulators. The transgenic event appears to be a single transgene insertion. Seeds representing this transformation event have been deposited with the American Type Culture Collection and have been assigned ATCC Accession No. _____.

Single seeds from plants that appeared to be homozygous for the transgene in pRVF7 were analyzed for fatty acid profile at maturity. All seeds accumulated vernolic acid with a mean content of 4.3% and a standard deviation of 1.2% between single seeds. Seeds from plants found not to contain the transgene by Southern analysis did not accumulate vernolic acid.

In soybean as in *Arabidopsis*, the gene sequence in SEQ ID NO:3 is capable of conferring the ability to accumulate vernolic acid on seeds of plants when those plants are transformed with a chimeric gene containing the sequence under the control of a suitable, seed specific promoter.

EXAMPLE 8

Expression of Chimeric Genes in Microbial Cells

The cDNAs encoding the instant Vernonia galamenensis fatty acid epoxidizing or desaturase enzymes can be inserted into the T7 E. coli expression vector pET24d (Novagen). For example, plasmid DNA containing a cDNA may be appropriately digested to release a nucleic acid fragment encoding the Vernonia galamenensis fatty acid epoxidizing enzyme. This fragment may then be purified on a 1% NuSieve GTG™ low melting agarose gel (FMC). Buffer and agarose contain 10 μg/ml ethidium bromide for visualization of the DNA fragment. The fragment can then be purified from the agarose gel by digestion with GELase™ (Epicentre Technologies) according to the manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 μL of water. Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase (New England Biolabs, Beverly, MA).

The fragment containing the ligated adapters can be purified from the excess adapters using low melting agarose as described above. The vector pET24d is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized with phenol/chloroform as decribed above. The prepared vector pET24d and fragment can then be ligated at 16°C for 15 hours followed by transformation into DH5 electrocompetent cells (GIBCO BRL). Transformants can be selected on agar plates containing 2xYT media and 50 μg/mL kanamycin. Transformants containing the gene are then screened for the correct orientation with respect to pET24d T7 promoter by restriction enzyme analysis.

Clones in the correct orientation with respect to the T7 promoter can be transformed into BL21(DE3) competent cells (Novagen) and selected on 2xYT agar plates containing 50 μg/ml kanamycin. A colony arising from this transformation construct can be grown overnight at 30°C in 2xYT media with 50 μg/mL kanamycin. The culture is then diluted two fold with fresh media, allowed to re-grow for 1 h, and induced by adding isopropyl-thiogalactopyranoside to 1 mM final concentration. Cells are then harvested by centrifugation after 3 h and re-suspended in 50 μL of 50 mM Tris-HCl at pH 8.0 containing 0.1 mM DTT and 0.2 mM phenyl methylsulfonyl fluoride. A small amount of 1 mm glass beads can be added and the mixture sonicated 3 times for about 5 seconds each time with a microprobe sonicator. The mixture is centrifuged and the protein concentration of the supernatant determined. One μg of protein from the soluble fraction of the culture can be separated by SDS-polyacrylamide gel electrophoresis. Gels can be observed for protein bands migrating at the expected molecular weight.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: USA
(F) ZIP: 19898
(G) TELEPHONE: 302-992-4926
(H) TELEFAX: 302-773-0164
(I) TELEX: 6717325

(ii) TITLE OF INVENTION: FATTY ACID MODIFYING ENZYMES FROM DEVELOPING SEEDS OF VERNONIA GALAMENENSIS

(iii) NUMBER OF SEQUENCES: 10

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.50 INCH
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: MICROSOFT WINDOWS 95
(D) SOFTWARE: MICROSOFT WORD VERSION 7.0A

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/872,302
(B) FILING DATE: JUNE 11, 1997

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: MAJARIAN, WILLIAM R.
(B) REGISTRATION NUMBER: 41,173
(C) REFERENCE/DOCKET NUMBER: BB-1084-A

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1476 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 134..1279

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GGCACGAGCT TCGCAGGCAC AGAGAAGGAA ATTGAGCGAT TAATCGCTTC TCCGAAGTGG      60

TTGTTTCTCC AGTGCAAACC CTAGGACTCC GTATATCGAT CGAATTAGGT TGAAGTGTCT     120

CCAGAACAAC AAA ATG GGA GCA GGA GGG TGC ATG TCT GCC TCC GAG ACA         169
               Met Gly Ala Gly Gly Cys Met Ser Ala Ser Glu Thr
                 1               5                  10

AAA ACA CAA CAA AAG AAC CCT ATC GAG CGA GTC CCT TAT GCA AAA CCT       217
Lys Thr Gln Gln Lys Asn Pro Ile Glu Arg Val Pro Tyr Ala Lys Pro
         15                  20                  25

CCT TTC ACC ATC AGC GAC CTC AAA AAA GCC ATT CCT CCC CAC TGT TTC       265
Pro Phe Thr Ile Ser Asp Leu Lys Lys Ala Ile Pro Pro His Cys Phe
     30                  35                  40

CAG CGT TCC CTT ATC CGT TCC TTC TCT TAT GTC GTT TAT GAC CTT GCT       313
Gln Arg Ser Leu Ile Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ala
 45                  50                  55                  60

GTG AGC TTC CTC CTC TAC TAT GTA GCC GCC ACT TAC TTC CAC CAT CTG       361
Val Ser Phe Leu Leu Tyr Tyr Val Ala Ala Thr Tyr Phe His His Leu
                 65                  70                  75

CCA AAC CCT TTC TCC TCC CTT GCG TGG CTG GCT TAT TGG GTC GTT CAA       409
Pro Asn Pro Phe Ser Ser Leu Ala Trp Leu Ala Tyr Trp Val Val Gln
             80                  85                  90

GGC TGT GTG CTT ACA GGA GTG TGG GTC ATA GCC CAT GAA TGT GGT CAC       457
Gly Cys Val Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His
         95                  100                 105

CAT GCA TTT AGT GAC TAT CAA TGG GTT GAT GAC ACT GTG GGC TTC CTA       505
His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Thr Val Gly Phe Leu
    110                 115                 120

CTC CAC TCG GTT CTA CTT GTT CCT TTC TTT TCA TGG AAA TAC AGT CAT       553
Leu His Ser Val Leu Leu Val Pro Phe Phe Ser Trp Lys Tyr Ser His
125                 130                 135                 140

CGT CGA CAC CAC TCC AAC ACC GGA TCA CTC GAG CGT GAT GAA GTC TTT       601
Arg Arg His His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe
                145                 150                 155

GTC CCA AAA CCG AGA TCG AAA ATC CCT TGG TAC TCA AAA TAC TTT AAC       649
Val Pro Lys Pro Arg Ser Lys Ile Pro Trp Tyr Ser Lys Tyr Phe Asn
            160                 165                 170
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AAC GCA CCT GGC CGC ATG ATG AGT GTG TTC ACC ACC CTA ACG CTA GGC          697
Asn Ala Pro Gly Arg Met Met Ser Val Phe Thr Thr Leu Thr Leu Gly
        175                 180                 185

TGG CCC TTG TAC TTG GTT TTC AAT GTA TCA GGG AGA CCC TAT GAC CGT          745
Trp Pro Leu Tyr Leu Val Phe Asn Val Ser Gly Arg Pro Tyr Asp Arg
    190                 195                 200

TTT GCC TGC CAC TTT TCT CCT AAC AGC CCT ATA TAC AAC GAA CGT GAG          793
Phe Ala Cys His Phe Ser Pro Asn Ser Pro Ile Tyr Asn Glu Arg Glu
205                 210                 215                 220

CGT CTC CAA ATA TGG CTT TCG GAT TTA GGG ATG ATC ACC ATG TCG TTC          841
Arg Leu Gln Ile Trp Leu Ser Asp Leu Gly Met Ile Thr Met Ser Phe
                225                 230                 235

ATC CTT TAT CGT GTT GCT GTA GCA AAA GGT GTG GCT TGG GTA ATA TGC          889
Ile Leu Tyr Arg Val Ala Val Ala Lys Gly Val Ala Trp Val Ile Cys
            240                 245                 250

ATG TAT GGG ATC CCG CTA CTG ATT GTG AAC GGA TTC CTG GTG ACG ATC          937
Met Tyr Gly Ile Pro Leu Leu Ile Val Asn Gly Phe Leu Val Thr Ile
        255                 260                 265

ACG TAC CTT CAA CAC ACT CAC CCT TCA TTG CCC CAC TAT GAT AGC TCA          985
Thr Tyr Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser
    270                 275                 280

GAG TGG GAC TGG CTA AGG GGA GCA ATG GCA ACG GTG GAC CGT GAC TAT         1033
Glu Trp Asp Trp Leu Arg Gly Ala Met Ala Thr Val Asp Arg Asp Tyr
285                 290                 295                 300

GGT GTG CTC AAC AAG GTA TTC CAT AAC ATC ACA GAT ACA CAC GTG GTG         1081
Gly Val Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Val
                305                 310                 315

CAC CAT TTG TTC TCG ACG ATG CCT CAT TAT AAC GCA ATG GAG GCA ACG         1129
His His Leu Phe Ser Thr Met Pro His Tyr Asn Ala Met Glu Ala Thr
            320                 325                 330

AAA GCA GTG AAG CCT TTG CTT GGG GAG TAT TAT CAG TTT GAC GGG ACT         1177
Lys Ala Val Lys Pro Leu Leu Gly Glu Tyr Tyr Gln Phe Asp Gly Thr
        335                 340                 345

CCG TTT TAC GTA GCA ATA TGG AGA GAG GCA AAG GAG TGT CTG TTC GTG         1225
Pro Phe Tyr Val Ala Ile Trp Arg Glu Ala Lys Glu Cys Leu Phe Val
    350                 355                 360

GAT CCA GAT GAG GGG GAG GGT CAG GGA GGT GTG TTT TGG TAC AAG AAT         1273
Asp Pro Asp Glu Gly Glu Gly Gln Gly Gly Val Phe Trp Tyr Lys Asn
365                 370                 375                 380

AAG ATG TGATATTCAT ATGATGAACA AGTTTTATAG GTTATCGATC AGATCAGGTC          1329
Lys Met

GGTTTATGTT GTTTATGTGC TTATGTGTGT GTGTGTTTTT TATGTGTAGC AAGCAGTTGA      1389

TCGAGTGTTG GGTATGTATG TGTCGTAGCG TCAATGAATT GAAAAGAACT GGTTATGTTC      1449

TTTAACATCA AAAAAAAAAA AAAAAAA                                          1476
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 382 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Gly Ala Gly Gly Cys Met Ser Ala Ser Glu Thr Lys Thr Gln Gln
 1               5                  10                  15

Lys Asn Pro Ile Glu Arg Val Pro Tyr Ala Lys Pro Pro Phe Thr Ile
            20                  25                  30

Ser Asp Leu Lys Lys Ala Ile Pro Pro His Cys Phe Gln Arg Ser Leu
        35                  40                  45

Ile Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ala Val Ser Phe Leu
    50                  55                  60

Leu Tyr Tyr Val Ala Ala Thr Tyr Phe His His Leu Pro Asn Pro Phe
65                  70                  75                  80

Ser Ser Leu Ala Trp Leu Ala Tyr Trp Val Val Gln Gly Cys Val Leu
                85                  90                  95

Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe Ser
            100                 105                 110

Asp Tyr Gln Trp Val Asp Asp Thr Val Gly Phe Leu Leu His Ser Val
        115                 120                 125

Leu Leu Val Pro Phe Phe Ser Trp Lys Tyr Ser His Arg Arg His His
    130                 135                 140

Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys Pro
145                 150                 155                 160

Arg Ser Lys Ile Pro Trp Tyr Ser Lys Tyr Phe Asn Asn Ala Pro Gly
                165                 170                 175

Arg Met Met Ser Val Phe Thr Thr Leu Thr Leu Gly Trp Pro Leu Tyr
            180                 185                 190

Leu Val Phe Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys His
        195                 200                 205

Phe Ser Pro Asn Ser Pro Ile Tyr Asn Glu Arg Glu Arg Leu Gln Ile
    210                 215                 220

Trp Leu Ser Asp Leu Gly Met Ile Thr Met Ser Phe Ile Leu Tyr Arg
225                 230                 235                 240

Val Ala Val Ala Lys Gly Val Ala Trp Val Ile Cys Met Tyr Gly Ile
                245                 250                 255

Pro Leu Leu Ile Val Asn Gly Phe Leu Val Thr Ile Thr Tyr Leu Gln
            260                 265                 270

His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp
        275                 280                 285

Leu Arg Gly Ala Met Ala Thr Val Asp Arg Asp Tyr Gly Val Leu Asn
    290                 295                 300
```

```
Lys Val Phe His Asn Ile Thr Asp Thr His Val Val His His Leu Phe
305                 310                 315                 320

Ser Thr Met Pro His Tyr Asn Ala Met Glu Ala Thr Lys Ala Val Lys
                325                 330                 335

Pro Leu Leu Gly Glu Tyr Tyr Gln Phe Asp Gly Thr Pro Phe Tyr Val
            340                 345                 350

Ala Ile Trp Arg Glu Ala Lys Glu Cys Leu Phe Val Asp Pro Asp Glu
        355                 360                 365

Gly Glu Gly Gln Gly Gly Val Phe Trp Tyr Lys Asn Lys Met
    370                 375                 380
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1364 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 103..1254

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TTATGAAAGC TCGATCGGTG TTCGATCAAT TCAAATCGAC GAACACGAAA TCGAACTCAA      60

CAATTCAAAT CTGGAAATAT TAATTGGATC AAGCGGGCGG AT ATG ATG ATG TCG      114
                                                 Met Met Met Ser
                                                   1

GAT TCA TGT GAT GAT CAT GAT CAG CTG GTG AAA GAT GAT CAT AAT ATA      162
Asp Ser Cys Asp Asp His Asp Gln Leu Val Lys Asp Asp His Asn Ile
  5                  10                  15                  20

AAC GAA CGT GCA CCG GTT GAT GCG GCA CCA TTC TCG TTA AGC GAT CTA      210
Asn Glu Arg Ala Pro Val Asp Ala Ala Pro Phe Ser Leu Ser Asp Leu
                25                  30                  35

AAG AAA GCA ATC CCT CCG CAT TGC TTC CAG CGA TCT GCC ATC CGT TCA      258
Lys Lys Ala Ile Pro Pro His Cys Phe Gln Arg Ser Ala Ile Arg Ser
            40                  45                  50

TCG TGC TAC GTT GTT CAG GAT CTC ATT ATT ACC TTC CTT TTA TAC ACG      306
Ser Cys Tyr Val Val Gln Asp Leu Ile Ile Thr Phe Leu Leu Tyr Thr
        55                  60                  65

CTC GCC AAC TCT TAC ATT CCT CTT CTT CCT CCT CCT CTA CCT TAC TTA      354
Leu Ala Asn Ser Tyr Ile Pro Leu Leu Pro Pro Pro Leu Pro Tyr Leu
    70                  75                  80

GCA TGG CCT GTT TAC TGG TTT TGC CAA TCT TCG ATC CTC ACT GGT TTA      402
Ala Trp Pro Val Tyr Trp Phe Cys Gln Ser Ser Ile Leu Thr Gly Leu
 85                  90                  95                 100

TGG GTC ATT GGC CAT GAA TGT GGC CAT CAT GCT TAT AGT GAG TAC CAG      450
Trp Val Ile Gly His Glu Cys Gly His His Ala Tyr Ser Glu Tyr Gln
                105                 110                 115
```

```
TGG GTT GAT AAC ACC GTT GGA TTC ATC CTC CAT TCC TTT CTT CTC ACA          498
Trp Val Asp Asn Thr Val Gly Phe Ile Leu His Ser Phe Leu Leu Thr
            120                 125                 130

CCT TAC TTT TCT TGG AAA TAC AGC CAT CGA AAG CAC CAT GCC AAC ACG          546
Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Lys His His Ala Asn Thr
        135                 140                 145

AAT TCA CTC GAA AAC GAG GAG GTT TAC ATT CCT AAA GCC AAG TCC CAG          594
Asn Ser Leu Glu Asn Glu Glu Val Tyr Ile Pro Lys Ala Lys Ser Gln
    150                 155                 160

CTC AGG AAT TAC TCC AAT TTC AAA TTT CTT GAC AAC ACC CCT GGT CGA          642
Leu Arg Asn Tyr Ser Asn Phe Lys Phe Leu Asp Asn Thr Pro Gly Arg
165                 170                 175                 180

ATC TTC ATT TTG CTT ATC ATG TTG ACC TTG GGC TTT CCT TTA TAC CTC          690
Ile Phe Ile Leu Leu Ile Met Leu Thr Leu Gly Phe Pro Leu Tyr Leu
                185                 190                 195

TTG ACC AAT ATT TCA GGC AAG AAA TAC CAA AGG TTT GCC AAC CAC TTT          738
Leu Thr Asn Ile Ser Gly Lys Lys Tyr Gln Arg Phe Ala Asn His Phe
            200                 205                 210

GAT CCG TTG AGC CCC ATC TTC AGT GAG CGT GAA CGA ATC CAG GTC GTG          786
Asp Pro Leu Ser Pro Ile Phe Ser Glu Arg Glu Arg Ile Gln Val Val
        215                 220                 225

CTA TCG GAT GTG GGT CTC ATT GCT GTG TTT TAC GGG CTT AAG TTT CTT          834
Leu Ser Asp Val Gly Leu Ile Ala Val Phe Tyr Gly Leu Lys Phe Leu
    230                 235                 240

GTA GCG AAA AAA GGG TTC GGT TGG GTA ATG CGC ATG TAC GGA GCC CCA          882
Val Ala Lys Lys Gly Phe Gly Trp Val Met Arg Met Tyr Gly Ala Pro
245                 250                 255                 260

GTG GTT GGG CTG AAT GCC TTC ATA ATA ATG ATC ACT TAT CTC CAC CAC          930
Val Val Gly Leu Asn Ala Phe Ile Ile Met Ile Thr Tyr Leu His His
                265                 270                 275

ACC CAT CTG TCT TCG CCT CAT TAC GAT TCG ACC GAA TGG AAC TGG ATC          978
Thr His Leu Ser Ser Pro His Tyr Asp Ser Thr Glu Trp Asn Trp Ile
            280                 285                 290

AAA GGA GCC TTG ACT ACA ATC GAT AGA GAT TTC GGT CTC CTG AAT AGG         1026
Lys Gly Ala Leu Thr Thr Ile Asp Arg Asp Phe Gly Leu Leu Asn Arg
        295                 300                 305

GTG TTC CAT GAC GTC ACT CAC ACA CAC GTG TTG CAT CAT TTG TTC CCG         1074
Val Phe His Asp Val Thr His Thr His Val Leu His His Leu Phe Pro
    310                 315                 320

TAC ATT CCA CAT TAT CAT GCA AAG GAG GCG AGC GAC GCA ATA AAG CCG         1122
Tyr Ile Pro His Tyr His Ala Lys Glu Ala Ser Asp Ala Ile Lys Pro
325                 330                 335                 340

GTG TTA GGG GAG TAT CGG ATG ATC GAT AGG ACT CCG TTT TAC AAA GCA         1170
Val Leu Gly Glu Tyr Arg Met Ile Asp Arg Thr Pro Phe Tyr Lys Ala
                345                 350                 355

ATG TGG AGA GAG GCG AAG GAA TGC ATC TAC ATC GAG CCA GAT GAA GAT         1218
Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Ile Glu Pro Asp Glu Asp
            360                 365                 370
```

AAG AAG CAC AAA GGT GTC TAT TGG TAC CAT AAA ATG TGATACTGC 1264
Lys Lys His Lys Gly Val Tyr Trp Tyr His Lys Met
375 380

TGAGTACGTA GTACGTTGTA TGCTTTTGTA ACGTTTTGTA AGATAAATAA ATAAATCTTG 1324

AATGAAGATA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 1364

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 384 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Met Met Ser Asp Ser Cys Asp Asp His Asp Gln Leu Val Lys Asp
1 5 10 15

Asp His Asn Ile Asn Glu Arg Ala Pro Val Asp Ala Ala Pro Phe Ser
20 25 30

Leu Ser Asp Leu Lys Lys Ala Ile Pro Pro His Cys Phe Gln Arg Ser
35 40 45

Ala Ile Arg Ser Ser Cys Tyr Val Val Gln Asp Leu Ile Ile Thr Phe
50 55 60

Leu Leu Tyr Thr Leu Ala Asn Ser Tyr Ile Pro Leu Leu Pro Pro Pro
65 70 75 80

Leu Pro Tyr Leu Ala Trp Pro Val Tyr Trp Phe Cys Gln Ser Ser Ile
85 90 95

Leu Thr Gly Leu Trp Val Ile Gly His Glu Cys Gly His His Ala Tyr
100 105 110

Ser Glu Tyr Gln Trp Val Asp Asn Thr Val Gly Phe Ile Leu His Ser
115 120 125

Phe Leu Leu Thr Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Lys His
130 135 140

His Ala Asn Thr Asn Ser Leu Glu Asn Glu Glu Val Tyr Ile Pro Lys
145 150 155 160

Ala Lys Ser Gln Leu Arg Asn Tyr Ser Asn Phe Lys Phe Leu Asp Asn
165 170 175

Thr Pro Gly Arg Ile Phe Ile Leu Leu Ile Met Leu Thr Leu Gly Phe
180 185 190

Pro Leu Tyr Leu Leu Thr Asn Ile Ser Gly Lys Lys Tyr Gln Arg Phe
195 200 205

Ala Asn His Phe Asp Pro Leu Ser Pro Ile Phe Ser Glu Arg Glu Arg
210 215 220

Ile Gln Val Val Leu Ser Asp Val Gly Leu Ile Ala Val Phe Tyr Gly
225 230 235 240

Leu Lys Phe Leu Val Ala Lys Lys Gly Phe Gly Trp Val Met Arg Met
245 250 255

```
Tyr Gly Ala Pro Val Val Gly Leu Asn Ala Phe Ile Ile Met Ile Thr
            260                 265                 270

Tyr Leu His His Thr His Leu Ser Ser Pro His Tyr Asp Ser Thr Glu
        275                 280                 285

Trp Asn Trp Ile Lys Gly Ala Leu Thr Thr Ile Asp Arg Asp Phe Gly
    290                 295                 300

Leu Leu Asn Arg Val Phe His Asp Val Thr His Thr His Val Leu His
305                 310                 315                 320

His Leu Phe Pro Tyr Ile Pro His Tyr His Ala Lys Glu Ala Ser Asp
                325                 330                 335

Ala Ile Lys Pro Val Leu Gly Glu Tyr Arg Met Ile Asp Arg Thr Pro
            340                 345                 350

Phe Tyr Lys Ala Met Trp Arg Glu Ala Lys Glu Cys Ile Tyr Ile Glu
        355                 360                 365

Pro Asp Glu Asp Lys Lys His Lys Gly Val Tyr Trp Tyr His Lys Met
    370                 375                 380
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 387 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Gly Leu Ala Lys Glu Thr Thr Met Gly Gly Arg Gly Arg Val Ala
  1                 5                  10                  15

Lys Val Glu Val Gln Gly Lys Lys Pro Leu Ser Arg Val Pro Asn Thr
             20                  25                  30

Lys Pro Pro Phe Thr Val Gly Gln Leu Lys Lys Ala Ile Pro Pro His
         35                  40                  45

Cys Phe Gln Arg Ser Leu Leu Thr Ser Phe Ser Tyr Val Val Tyr Asp
     50                  55                  60

Leu Ser Phe Ala Phe Ile Phe Tyr Ile Ala Thr Thr Tyr Phe His Leu
 65                  70                  75                  80

Leu Pro Gln Pro Phe Ser Leu Ile Ala Trp Pro Ile Tyr Trp Val Leu
                 85                  90                  95

Gln Gly Cys Leu Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly
             100                 105                 110

His His Ala Phe Ser Lys Tyr Gln Trp Val Asp Asp Val Val Gly Leu
         115                 120                 125

Thr Leu His Ser Thr Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser
     130                 135                 140

His Arg Arg His His Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val
145                 150                 155                 160
```

Phe Val Pro Lys Pro Lys Ser Lys Val Ala Trp Phe Ser Lys Tyr Leu
165 170 175

Asn Asn Pro Leu Gly Arg Ala Val Ser Leu Leu Val Thr Leu Thr Ile
180 185 190

Gly Trp Pro Met Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp
195 200 205

Ser Phe Ala Ser His Tyr His Pro Tyr Ala Pro Ile Tyr Ser Asn Arg
210 215 220

Glu Arg Leu Leu Ile Tyr Val Ser Asp Val Ala Leu Phe Ser Val Thr
225 230 235 240

Tyr Ser Leu Tyr Arg Val Ala Thr Leu Lys Gly Leu Val Trp Leu Leu
245 250 255

Cys Val Tyr Gly Val Pro Leu Leu Ile Val Asn Gly Phe Leu Val Thr
260 265 270

Ile Thr Tyr Leu Gln His Thr His Phe Ala Leu Pro His Tyr Asp Ser
275 280 285

Ser Glu Trp Asp Trp Leu Lys Gly Ala Leu Ala Thr Met Asp Arg Asp
290 295 300

Tyr Gly Ile Leu Asn Lys Val Phe His His Ile Thr Asp Thr His Val
305 310 315 320

Ala His His Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala
325 330 335

Thr Asn Ala Ile Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Phe Asp Asp
340 345 350

Thr Pro Phe Tyr Lys Ala Leu Trp Arg Glu Ala Arg Glu Cys Leu Tyr
355 360 365

Val Glu Pro Asp Glu Gly Thr Ser Glu Lys Gly Val Tyr Trp Tyr Arg
370 375 380

Asn Lys Tyr
385

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 387 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Gly Gly Gly Gly Arg Met Ser Thr Val Ile Thr Ser Asn Asn Ser
1 5 10 15

Glu Lys Lys Gly Gly Ser Ser His Leu Lys Arg Ala Pro His Thr Lys
20 25 30

Pro Pro Phe Thr Leu Gly Asp Leu Lys Arg Ala Ile Pro Pro His Cys
35 40 45

```
Phe Glu Arg Ser Phe Val Arg Ser Phe Ser Tyr Val Ala Tyr Asp Val
     50                  55                  60

Cys Leu Ser Phe Leu Phe Tyr Ser Ile Ala Thr Asn Phe Phe Pro Tyr
 65                  70                  75                  80

Ile Ser Ser Pro Leu Ser Tyr Val Ala Trp Leu Val Tyr Trp Leu Phe
                 85                  90                  95

Gln Gly Cys Ile Leu Thr Gly Leu Trp Val Ile Gly His Glu Cys Gly
            100                 105                 110

His His Ala Phe Ser Glu Tyr Gln Leu Ala Asp Asp Ile Val Gly Leu
        115                 120                 125

Ile Val His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser
    130                 135                 140

His Arg Arg His His Ser Asn Ile Gly Ser Leu Glu Arg Asp Glu Val
145                 150                 155                 160

Phe Val Pro Lys Ser Lys Ser Lys Ile Ser Trp Tyr Ser Lys Tyr Ser
                165                 170                 175

Asn Asn Pro Pro Gly Arg Val Leu Thr Leu Ala Ala Thr Leu Leu Leu
            180                 185                 190

Gly Trp Pro Leu Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp
        195                 200                 205

Arg Phe Ala Cys His Tyr Asp Pro Tyr Gly Pro Ile Phe Ser Glu Arg
    210                 215                 220

Glu Arg Leu Gln Ile Tyr Ile Ala Asp Leu Gly Ile Phe Ala Thr Thr
225                 230                 235                 240

Phe Val Leu Tyr Gln Ala Thr Met Ala Lys Gly Leu Ala Trp Val Met
                245                 250                 255

Arg Ile Tyr Gly Val Pro Leu Leu Ile Val Asn Cys Phe Leu Val Met
            260                 265                 270

Ile Thr Tyr Leu Gln His Thr His Pro Ala Ile Pro Arg Tyr Gly Ser
        275                 280                 285

Ser Glu Trp Asp Trp Leu Arg Gly Ala Met Val Thr Val Asp Arg Asp
    290                 295                 300

Tyr Gly Val Leu Asn Lys Val Phe His Asn Ile Ala Asp Thr His Val
305                 310                 315                 320

Ala His His Leu Phe Ala Thr Val Pro His Tyr His Ala Met Glu Ala
                325                 330                 335

Thr Lys Ala Ile Lys Pro Ile Met Gly Glu Tyr Tyr Arg Tyr Asp Gly
            340                 345                 350

Thr Pro Phe Tyr Lys Ala Leu Trp Arg Glu Ala Lys Glu Cys Leu Phe
        355                 360                 365

Val Glu Pro Asp Glu Gly Ala Pro Thr Gln Gly Val Phe Trp Tyr Arg
    370                 375                 380

Asn Lys Tyr
385
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGCGGCCGC ATGATGATGT CGGATTCATG 30

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATGCGGCCGC TCACATTTTA TGGTACCAAT AT 32

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATGCGGCCGC CATGGGAGCA GGAGGG 26

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATGCGGCCGC TCACATCTTA TTCTTGTACC AA 32

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 5, line 6

B. **IDENTIFICATION OF DEPOSIT** Further deposits are identified on an additional sheet ☐

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*

10801 University Blvd.
Manassas, Virginia 20110-2209
USA

Date of deposit	Accession Number

C. **ADDITIONAL INDICATIONS** *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. **DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** *(if the indications are not for all designated States)*

E. **SEPARATE FURNISHING OF INDICATIONS** *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

Accession number and number of deposit will be submitted later

For receiving Office use only	For International Bureau use only
☑ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer Sonya Barnes PCT International Division	Authorized officer

CLAIMS

What is claimed is:

1. An isolated nucleic acid fragment encoding a *Vernonia galamenensis* fatty acid desaturse comprising a member selected from the group consisting of:

(a) an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in SEQ ID NO:2;

(b) an isolated nucleic acid fragment that is substantially similar to an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in SEQ ID NO:2; and

(c) an isolated nucleic acid fragment that is complementary to (a) or (b).

2. The isolated nucleic acid fragment of Claim 1 wherein the nucleotide sequence of the fragment is set forth in SEQ ID NO:1.

3. A chimeric gene comprising the nucleic acid fragment of Claim 1 operably linked to suitable regulatory sequences.

4. A transformed host cell comprising the chimeric gene of Claim 3.

5. An isolated nucleic acid fragment encoding a *Vernonia galamenensis* fatty acid epoxidizing enzyme comprising a member selected from the group consisting of:

(a) an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in SEQ ID NO:4;

(b) an isolated nucleic acid fragment that is substantially similar to an isolated nucleic acid fragment encoding all or a substantial portion of the amino acid sequence set forth in SEQ ID NO:4; and

(c) an isolated nucleic acid fragment that is complementary to (a) or (b).

6. The isolated nucleic acid fragment of Claim 5 wherein the nucleotide sequence of the fragment is set forth in SEQ ID NO:3.

7. A chimeric gene comprising the nucleic acid fragment of Claim 5 operably linked to suitable regulatory sequences.

8. A transformed host cell comprising the chimeric gene of Claim 7.

9. A transformed plant comprising the host cell of Claim 8.

10. A seed from the transformed plant of Claim 9 wherein the seed comprises the chimeric gene of Claim 7.

11. A soybean seed that accumulates vernolic acid.

12. The soybean seed of Claim 11 wherein said seed bears ATCC Accession No. _____.

13. A method of altering the level of expression of a *Vernonia galamenensis* fatty acid modifying enzyme in a host cell comprising:

(a) transforming a host cell with the chimeric gene of Claim 3 or the chimeric gene of Claim 7; and

(b) growing the transformed host cell produced in step (a) under conditions that are suitable for expression of the chimeric gene

wherein expression of the chimeric gene results in production of altered levels of a *Vernonia galamenensis* fatty acid modifying enzyme in the transformed host cell.

14. A method for producing epoxidized fatty acids in the seeds of plants comprising the following steps:

(a) transforming a plant cell with a chimeric gene encoding all or a portion of a *Vernonia galamenensis* fatty acid epoxidizing enzyme operably linked in sense orientation to suitable regulatory sequences;

(b) growing a fertile mature plant from the transformed plant cell of step (a) under conditions suitable to obtain seeds; and

(c) selecting from the progeny seed of step (b) those seeds containing epoxidized fatty acids.

15. A method for producing a *Vernonia galamenensis* fatty acid modifying enzyme comprising the following steps:

(a) transforming a microbial host cell with a chimeric gene wherein a nucleic acid fragment encoding a *Vernonia galamenensis* fatty acid modifying enzyme is operably linked to regulatory sequences suitable for directing expression in microbial cells; and

(b) growing the transformed microbial cells obtained from step (a) under conditions that result in expression of the *Vernonia galamenensis* fatty acid modifying enzyme protein.

16. A method of obtaining a nucleic acid fragment encoding all or substantially all of the amino acid sequence encoding a *Vernonia galamenensis* fatty acid modifying enzyme comprising:

(a) probing a cDNA or genomic library with the nucleic acid fragment of Claim 1 or Claim 5;

(b) identifying a DNA clone that hybridizes to the nucleic acid fragment of Claim 1 or Claim 5; and

(c) isolating the DNA clone identified in step (b);

wherein the isolated DNA clone of step (c) comprises a nucleic acid fragment encoding all or substantially all of the amino acid sequence encoding a *Vernonia galamenensis* fatty acid modifying enzyme.

17. A method of obtaining a nucleic acid fragment encoding a portion of an amino acid sequence encoding a *Vernonia galamenensis* fatty acid modifying enzyme comprising:

(a) synthesizing an oligonucleotide primer corresponding to a portion of the sequence set forth in a member selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:3; and

(b) amplifying a cDNA insert present in a cloning vector using the oligonucleotide primer of step (a) and a primer representing sequences of the cloning vector

wherein the amplified nucleic acid fragment encodes a portion of an amino acid sequence encoding a *Vernonia galamenensis* fatty acid modifying enzyme.

18. The product of the method of Claim 16.

19. The product of the method of Claim 17.

FIG.1

```
1   MGLAKETTMGGRGRVAKVEVQGKKPLSRVPNTKPPFTVGQLKKAIPPHCFQRSLLTSFSY soy
1   ------MGAGGCMSASETKTQQKNPIERVPYAKPPFTISDLKKAIPPHCFQRSLIRSFSY vs1.05h08
1   -----MMMSDSCDDHDQLVKDDHNINERAPVDAAPFSLSDLKKAIPPHCFQRSAIRSSCY vs1.02c07
1   -MGGGGRMSTVITSNNSEKKGGSSHLKRAPHTKPPFTLGDLKRAIPPHCFERSFVRSFSY castor

61  VVYDLSFAFI-FYIATTYFHLLPQPFSLIAWPIYWVLQGCLLTGVWVIAHECGHHAFSKY soy
55  VVYDLAVSFLLYYVAATYFHHLPNPFSSLAWLAYWVVQGCVLTGVWVIAHECGHHAFSDY vs1.05h08
56  VVQDLIITFLLYTLANSYIPLLPPPLPYLAWPVYWFCQSSILTGLWVIGHECGHHAYSEY vs1.02c07
60  VAYDVCLSFLFYSIATNFFPYISSPLSYVAWLVYWLFQGCILTGLWVIGHECGHHAFSEY castor

120 QWVDDVVGLTLHSTLLVPYFSWKISHRRHHSNTGSLDRDEVFVPKPKSKVAWFS--KYLN soy
115 QWVDDTVGFLLHSVLLVPFFSWKYSHRRHHSNTGSLERDEVFVPKPRSKIPWYS--KYFN vs1.05h08
116 QWVDNTVGFILHSFLLTPYFSWKYSHRKHHANTNSLENEEVYIPKAKSQLRNYSNFKFLD vs1.02c07
120 QLADDIVGLIVHSALLVPYFSWKYSHRRHHSNIGSLERDEVFVPKSKSKISWYS--KYSN castor

178 NPLGRAVSLLVTLTIGWPMYLAFNVSGRPYDSFASHYHPYAPIYSNRERLLIYVSDVALF soy
173 NAPGRMMSVFTTLTLGWPLYLVFNVSGRPYDRFACHFSPNSPIYNERERLQIWLSDLGMI vs1.05h08
176 NTPGRIFILLIMLTLGFPLYLLTNISGKKYQRFANHFDPLSPIFSERERIQVVLSDVGLI vs1.02c07
178 NPPGRVLTLAATLLLGWPLYLAFNVSGRPYDRFACHYDPYGPIFSERERLQIYIADLGIF castor

238 SVTYSLYRVATLKGLVWLLCVYGVPLLIVNGFLVTITYLQHTHFALPHYDSSEWDWLKGA soy
233 TMSFILYRVAVAKGVAWVICMYGIPLLIVNGFLVTITYLQHTHPSLPHYDSSEWDWLRGA vs1.05h08
236 AVFYGLKFLVAKKGFGWVMRMYGAPVVGLNAFIIMITYLHHTHLSSPHYDSTEWDWIKGA vs1.02c07
238 ATTFVLYQATMAKGLAWVMRIYGVPLLIVNCFLVMITYLQHTHPAIPRYGSSEWDWLRGA castor

298 LATMDRDYGILNKVFHHITDTHVAHHLFSTMPHYHAMEATNAIKPILGEYYQFDDTPFYK soy
293 MATVDRDYGVLNKVFHNITDTHVVHHLFSTMPHYNAMEATKAVKPLLGEYYQFDGTPFYV vs1.05h08
296 LTTIDRDFGLLNRVFHDVTHTHVLHHLFPYIPHYHAKEASDAIKPVLGEYRMIDRTPFYK vs1.02c07
298 MVTVDRDYGVLNKVFHNIADTHVAHHLFATVPHYHAMEATKAIKPIMGEYYRYDGTPFYK castor

358 ALWREARECLYVEPDEGTSEKGVYWYRNKY.                                 soy
353 AIWREAKECLFVDPDEGEGQGGVFWYKNKM.                                 vs1.05h08
356 AMWREAKECIYIEPDEDKKHKGVYWYH-KM.                                 vs1.02c07
358 ALWREAKECLFVEPDEGAPTQGVFWYRNK-Y                                 castor
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FIGURE 2

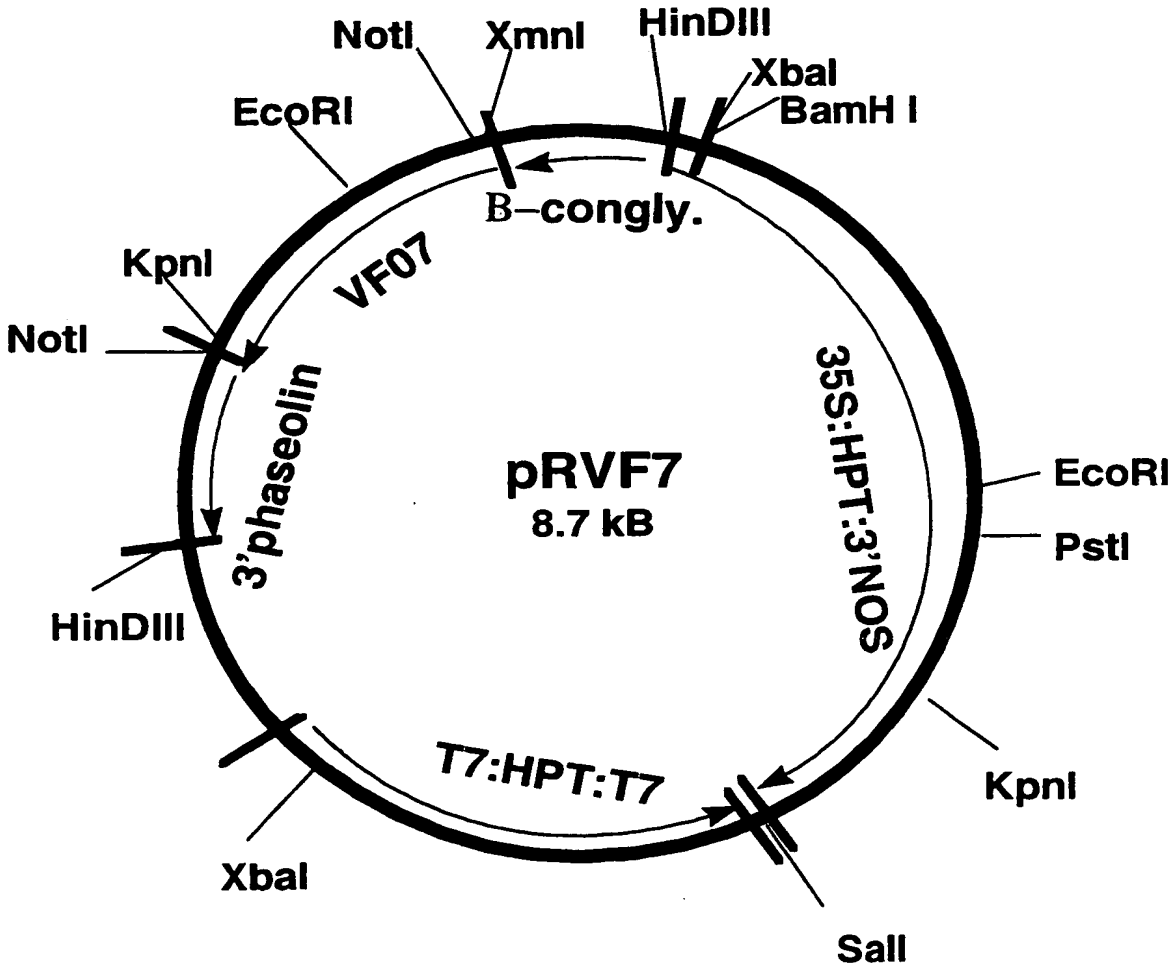
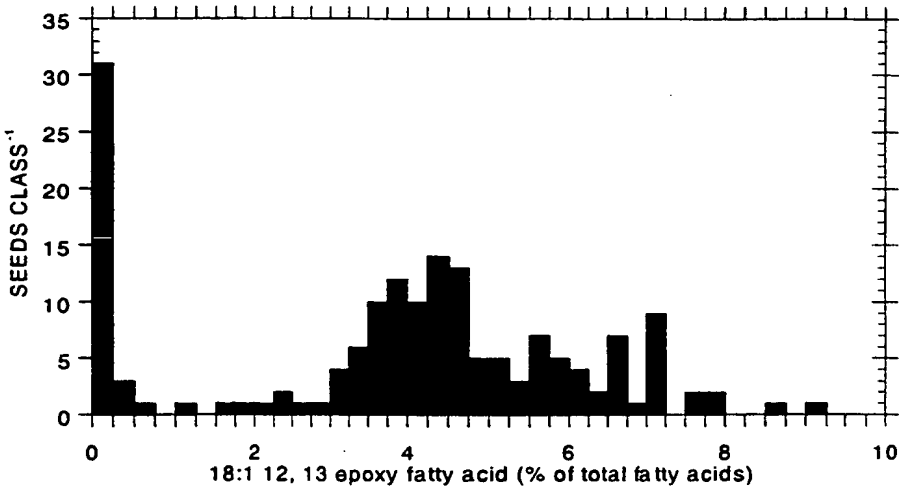


FIGURE 3



Applicant's or agent's file reference number BB10[illegible] | International application No. PCT/US98/12074

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 5, line 6

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
10801 University Blvd.
Manassas, Virginia 20110-2209
USA

Date of deposit	Accession Number
13 June 1998 (13.06.98)	209967

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

Accession and Number of Deposit will be submitted later

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INTERNATIONAL SEARCH REPORT

[redacted] onal Application No
[redacted] T/US 98/12074

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/11 C12N15/82 C12N1/21 C12N5/10
C12N9/02 C12Q1/68 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SEITHER G. ET AL.: "Isolation of cytochrome P-450 genes from Vernonia galamensis" PHYSIOL. BIOCHEM. MOL. BIOL. PLANT LIPIDS (PROC. INT. SYMP. PLANT LIPIDS),1997, pages 389-391, XP002080169 see the whole document ---	5,7-10, 15,16
A	BAFOR M. ET AL.: "Biosynthesis of vernoleate (cis-12-epoxyoctadeca-cis-9-enoate) in microsomal preparations from developing endosperm of Euphorbia lagascae" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 303, no. 1, 15 May 1993, pages 145-151, XP002080170 cited in the application see the whole document ---	1-19

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 1998	22/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Kania, T

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INTERNATIONAL SEARCH REPORT

Inter[redacted]l Application No
PCT[redacted]98/12074

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 11516 A (DU PONT ;LIGHTNER JONATHAN EDWARD (US); OKULEY JOHN JOSEPH (US)) 26 May 1994 cited in the application see the whole document ---	1-19
A	US 5 430 134 A (OHLROGGE JOHN B ET AL) 4 July 1995 see the whole document -----	1-19

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INTERNATIONAL SEARCH REPORT

[redacted]mation on patent family members

[redacted]onal Application No
[redacted]T/US 98/12074

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9411516 A	26-05-1994	AU 5407594 A	08-06-1994
		CA 2149223 A	26-05-1994
		EP 0668919 A	30-08-1995
		JP 8503364 T	16-04-1996
US 5430134 A	04-07-1995	NONE	